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Regulation of Excitation-Contraction and Excitation-Transcription Coupling in Gastrointestinal Smooth Muscle by Caveolin-1

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**Regulation of Excitation-Contraction and Excitation-Transcription Coupling
in Gastrointestinal Smooth Muscle by Caveolin-1**

**A thesis submitted in partial fulfilment of the requirements for the
degree of Doctor of Philosophy at Virginia Commonwealth University**

by

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Table of content

Acknowledgment	ii
Table of Content	iv
List of Tables.....	ix
List of Figures	x
List of Abbreviations.....	xiii
Abstract	xv
CHAPTER I. INTRODUCTION	1
I. Structure of the GI tract.....	1
I.1. Enteric nervous system.....	2
I.1a. Motor neurons.....	2
I.1b. Interneurons.....	3
I.1c. Sensory neurons.....	3
I.1d. Neurotransmitters in the gastrointestinal tract.....	4
I.2. Interstitial Cells of Cahal (ICCs).....	7
I.3. Gastrointestinal smooth muscle and its contractile apparatus.....	8
I.4. Signaling mechanisms involved in smooth muscle contraction.....	9
I.4.1. Regulation of myosin light chain kinase (MLCK).....	10
I.4.2. Regulation of myosin light chain phosphatase.....	11
I.5. Signaling pathways involved in smooth muscle relaxation.....	13
I.5.1. Regulation of cyclic nucleotide levels.....	14
I.5.2. Molecular targets of PKA and PKG.....	15
I.6. Receptor desensitization and internalization.....	16

I.6.1. Molecular mechanism of homologous desensitization.....	17
I.6.2. Molecular mechanism of heterologous desensitization.....	18
I.6.3. Deactivation of G-protein by RGS proteins.....	19
I.7. GPCR internalization.....	20
I.8. Regulation of GPCR signaling by scaffolding proteins.....	20
I.8.1.A-kinase anchoring proteins (AKAPs).....	21
I.8.2. PDZ-domain containing scaffolding proteins.....	22
I.8.3. Tetratricopeptide repeat (TPR)-bearing scaffolding proteins.....	22
I.8.4. β -arrestins.....	23
I.8.5. Caveolin proteins.....	24
I.8.5a. Regulation of endothelial nitric oxide synthase by caveolin.....	25
I.8.5b. Regulation of cAMP/cGMP signaling by caveolins.....	26
I.8.5c. Regulation of receptor tyrosine kinases by caveolins.....	26
I.8.5d. Regulation of ion channels by caveolins.....	27
I.8.5e. Regulation of endocytosis by caveolin proteins.....	28
I.9. Regulation of smooth muscle function by caveolins.....	28
I.10. Novelty and significance.....	31
CHAPTER II. MATERIALS AND METHODS.....	33
II.1. Materials.....	33
II.2. Methods.....	36
II.2.1. Tissue collection and processing.....	36
II.2.2. Preparation of gastric smooth muscle cells.....	36
II.2.3. Preparation of primary cultures of gastric muscle cells.....	37

II.2.4. Total RNA isolation.....	38
II.2.5. Real time PCR.....	38
II.2.6. Western blot analysis.....	41
II.2.7. Radioligand binding studies.....	42
II.2.8. Assay for phosphoinositide hydrolysis.....	42
II.2.9. Assay for adenylyl cyclase activity.....	43
II.2.10. Assay for Rho kinase and ZIP kinase activity.....	44
II.2.11. Transfection of caveolin-1 siRNA into cultured gastric cells.....	45
II.2.12. Sucrose density centrifugation to isolate caveolar and non-caveolar fractions.....	46
II.2.13. Receptor internalization by radioligand binding studies.....	47
II.2.14. Measurement of muscle cell contraction by scanning micrometry.....	48
II.2.15. In-cell western.....	48
II.3. Statistical analysis.....	50
CHAPTER III. RESULTS.....	51
III.1. Signaling transduction by muscarinic receptors.....	51
III.1a. Expression of muscarinic m2 and m3 receptors in gastric smooth muscle.....	51
III.2. Signaling pathways coupled to m2 and m3 receptors in gastric smooth muscle.....	53
III.2a. G _q -coupled muscarinic m3 receptor signalling.....	53
III.2b. G ₁₃ -coupled muscarinic m3 receptor signalling.....	54
III.2c. G _i - coupled muscarinic m3 receptor signalling.....	55
III.2d. m3 mediated muscle contraction.....	56

III.3. Regulation of G _q -coupled m3 receptor signaling by caveolae and caveolin-1.....	73
III.3a. Inhibition of carbachol-stimulated PI hydrolysis by methyl β-cyclodextrin.....	73
III.3b. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 siRNA.....	74
III.3c. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 KO.....	75
III.3d. Inhibition of carbachol-stimulated initial MLC ₂₀ phosphorylation and muscle contraction by MβCD and caveolin-1 KO.....	76
III.4. Regulation of G ₁₃ -coupled m3 receptor signaling by caveolae and caveolin-1.....	90
III.4a. Inhibition of carbachol-stimulated Rho kinase activity by MβCD.....	90
III.4b. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 siRNA.....	91
III.4c. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 KO.....	91
III.4d. Inhibition of carbachol-stimulated ZIP kinase activity by MβCD.....	92
III.4e. Inhibition of carbachol-stimulated ZIP kinase activity by caveolin-1 siRNA.....	93
III.4f. Inhibition of carbachol-stimulated ZIP kinase activity by caveolin-1 KO.....	93
III.4g. Inhibition of carbachol-stimulated MYPT1 phosphorylation and muscle contraction by MβCD and caveolin-1 KO.....	94
III.5. Regulation of G _i -coupled m2 receptor signaling by caveolae and caveolin-1.....	113
III.5a. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by methyl β-cyclodextrin.....	113
III.5b. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by caveolin-1 siRNA.....	114

III.5c. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by caveolin-1 KO.....	115
III.6. Regulation of muscarinic m2 and m3 receptor internalization by caveolin-1.....	123
III.6a. Inhibition of m2 receptor internalization by methyl β -cyclodextrin.....	124
III.6b. Inhibition of m2 receptor internalization by caveolin-1 siRNA.....	125
III.6c. Inhibition of m2 receptor internalization by Src kinase inhibitor, PP2.....	126
III.6d. Lack of effect on m3 receptor internalization by methyl β -cyclodextrin.....	126
III.6e. Lack of effect on m3 receptor internalization by caveolin-1 siRNA.....	126
III.6f. Lack of effect on m3 receptor internalization by Src kinase inhibitor, PP2.....	128
III.7. Regulation of excitation-transcription coupling by caveolin-1.....	146
CHAPTER IV. DISCUSSION.....	158
IV.1. Signaling transduction by muscarinic receptors.....	160
IV.2. Regulation of m2 and m3 receptor signaling by caveolae and caveolin-1.....	163
IV.2a. Regulation of G_q -coupled m3 receptor signaling by caveolin-1.....	165
IV.2b. Regulation of G_{13} -coupled m3 receptor signaling by caveolin-1.....	167
IV.2c. Regulation of G_i -coupled m2 receptor signaling by caveolin-1.....	170
IV.3. Regulation of m2 and m3 receptor internalization by caveolae and caveolin-1.....	172
IV.3a. Regulation of m2 receptor internalization by caveolae and caveolin-1.....	173
IV.3b. Regulation of m3 receptor internalization by caveolae and caveolin-1.....	175
IV.4. Regulation of Excitation-transcription coupling by caveolin-1.....	176
REFERENCES.....	182

LIST OF TABLES

	Page
Table 1. Real-time and RT-PCR primer sequences.....	35

LIST OF FIGURES

Figure 1. Selective expression of muscarinic m2 and m3 receptors in gastric smooth muscle cells.....	57
Figure 2. Selective expression of muscarinic m2 and m3 receptors by radioligand binding studies.....	59
Figure 3. m3 receptor-dependent stimulation of phosphoinositide (PI) hydrolysis in gastric muscle.....	61
Figure 4. m3 receptor-dependent stimulation of Rho Kinase activity in gastric muscle	63
Figure 5. m3 receptor-dependent stimulation of zipper interacting protein (ZIP) Kinase activity in gastric muscle	65
Figure 6. m2 receptor-dependent inhibition of adenylyl cyclase activity in gastric muscle	67
Figure 7. m3 receptor-dependent muscle contraction in gastric muscle.....	69
Figure 8. Signaling pathways activated by m2 and m3 receptors in gastric muscle cells.....	71
Figure 9. Expression of caveolin-1 and -3 in gastric smooth muscle.....	78
Figure 10. Inhibition of carbachol-stimulated PI hydrolysis by M β CD	80
Figure 11. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 siRNA.....	82
Figure 12. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 KO.....	84
Figure 13. Inhibition of carbachol-stimulated initial MLC ₂₀ phosphorylation by M β CD.....	86

Figure 14. Inhibition of carbachol-stimulated initial muscle contraction by M β CD and caveolin-1 KO	88
Figure 15. Inhibition of carbachol-stimulated Rho Kinase activity by M β CD.....	97
Figure. 16. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 siRNA.....	99
Figure 17. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 KO.....	101
Figure 18. Inhibition of carbachol-stimulated zipper interacting protein (ZIP) kinase activity by M β CD.....	103
Figure 19. Inhibition of carbachol-stimulated zipper interacting protein (ZIP) kinase activity by caveolin-1 siRNA.....	105
Figure 20. Inhibition of carbachol-stimulated zipper interacting protein (ZIP) kinase activity by caveolin-1 KO.....	107
Figure 21. Inhibition of carbachol-stimulated MYPT1 phosphorylation by M β CD.....	109
Figure 22. Inhibition of carbachol-stimulated sustained muscle contraction by M β CD and caveolin-1 KO.....	111
Figure 23. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by M β CD...	117
Figure 24. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by caveolin-1 siRNA.....	119
Figure 25. Lack of effect on m2-mediated inhibition of adenylyl cyclase (AC) activity by caveolin-1 KO.....	121
Figure 26. Localization of muscarinic m2 and m3 receptors in caveolar and non-caveolar fractions.....	130
Figure 27. Pathways for receptor internalization.....	132
Figure 28. Inhibition of m2 receptor internalization by M β CD.....	134

Figure 29. Inhibition of m2 receptor internalization by caveolin-1 siRNA.....	136
Figure 30. Inhibition of m2 receptor internalization by Src kinase inhibitor, PP2.....	138
Figure 31. Lack of effect on m3 receptor internalization by M β CD.....	140
Figure 32. Lack of effect on m3 receptor internalization by caveolin-1 siRNA.....	142
Figure 33. Lack of effect on m3 receptor internalization by Src inhibitor, PP2.....	144
Figure 34. Excitation-contraction coupling in smooth muscle.....	148
Figure 35. Inhibition of serum response factor (SRF) and myocardin expression by caveolin-1 siRNA.....	150
Figure 36. Inhibition of γ -actin and caldesmon expression by caveolin-1 siRNA.....	152
Figure 37. Augmentation of EGF receptor phosphorylation by caveolin-1 siRNA.....	154
Figure 38. Augmentation of ERK1/2 activity by caveolin-1 siRNA.....	156

List of Abbreviations

5-HT.....	Serotonin
4-DAMP.....	1,1-Dimethyl-4-diphenylacetoxypiper idinium iodide
AC.....	Adenylate cyclase
Ach.....	Acetyl Choline
AMPK.....	Adenosine monophosphate kinase
BSA.....	Bovine serum albumin
CaD.....	Caldesmon
CaM.....	Calmodulin
CaMKII.....	Calmodulin-dependent kinase II
CaP.....	Calponin
Cav.....	caveolin
Cav-1, 2, and 3.....	caveolin-1, 2, and 3
CCh.....	Carbachol
c.p.m.....	counts per minute
CT.....	cycle threshold
DMEM.....	Dulbecco's Modified Eagle Medium
DMSO.....	Dimethyl sulfoxide
ENS.....	Enteric nervous system
Fsk.....	Forskolin

GAPDH.....	Glyceraldehyde 3-phosphate dehydrogenase
GI.....	Gastrointestinal
HEPES.....	N-2 hydroxyethylpiperazine-N' 2-ethanesulfonic acid
IBMX.....	3-isobutyl-1-methylxanthine
IP ₃	inositol triphosphate
KO.....	Knockout mice
MβCD.....	methyl b cyclodextrin
MBP.....	Myelin basic protein
MLC.....	Myosin light chain
MLCK.....	Myosin light chain kinase
MLCP.....	Myosin light chain phosphatase
MYPT1.....	Myosin phosphatase target subunit 1
NO.....	nitric oxide
PI.....	Phosphoinositide
PLC.....	Phospholipase C
ROCK.....	Rho Kinase
WT.....	Wild Type
ZIPK.....	Zipper interacting protein kinase

ABSTRACT

Caveolae are integral part of the smooth muscle membrane and caveolins, the defining proteins of caveolae, act as scaffolding proteins for several G protein-coupled receptor signaling molecules and regulate cellular signaling through direct and indirect interactions with signaling proteins. Caveolin-1 is the predominant isoform in the smooth muscle and drives the formation of caveolae. However, little is known about the role of caveolin-1 in the regulation of excitation-contraction and excitation-transcription coupling in gastrointestinal smooth muscle. In the present study we have characterized muscarinic m2 and m3 receptor signaling in gastric smooth muscle and tested the hypothesis that caveolin-1 positively regulates muscarinic receptor signaling and contractile protein expression in smooth muscle. The role of caveolae/caveolin-1 in the regulation of muscarinic signaling was examined using complementary approaches: a) methyl β -cyclodextrin (M β CD) to deplete cholesterol in dispersed muscle cells, b) caveolin-1 siRNA to suppress caveolin-1 expression in cultured muscle cells, and c) caveolin-1 knockout (KO) mice.

RT-PCR, western blot and radioligand binding studies demonstrated the selective expression of m2 and m3 receptor in gastric smooth muscle cells. Carbachol (CCh), acting via m3 receptors caused stimulation of phosphoinositide (PI) hydrolysis, Rho kinase and ZIP kinase activity, and induced phosphorylation of MYPT1 (at Thr⁶⁹⁶) and MLC₂₀ (at Ser¹⁹), and muscle contraction, and acting via m2 receptors caused inhibition of forskolin stimulated cAMP formation. Stimulation of PI hydrolysis, Rho kinase and ZIP kinase activities, phosphorylation of MYPT1 and MLC₂₀ phosphorylation and muscle contraction in response to CCh was attenuated

in dispersed cells treated with M β CD or in cultured cells transfected with caveolin-1 siRNA. Similar inhibition of all responses was obtained in gastric muscle cells from caveolin-1 KO mice compared to gastric muscle cells to WT mice. Although, caveolin-1 had no effect on m2 receptor signaling, agonist-induced internalization of m2, but not m3 receptors was blocked in dispersed cells treated with M β CD or in cultured cells transfected with caveolin-1 siRNA. These results suggest that caveolin-1 selectively and positively regulates G_{q/13}-coupled m3 receptor signaling, G_i-coupled m2 receptor internalization. The expression of contractile proteins, γ -actin and caldesmone and the transcription factors SRF and myocardin that regulate the expression of contractile proteins are down regulated, whereas EGF-stimulated EGF receptor phosphorylation and ERK1/2 activity are up-regulated in cells transfected with caveolin-1 siRNA. These results suggest using pharmacological, molecular and genetic approaches provide conclusive evidence that caveolae and caveolin-1 play an important role in orchestrating G protein coupled receptor signaling to have dual pro- excitation-contraction and excitation-transcription coupling, and anti-proliferative role in gastric smooth muscle.

CHAPTER I: INTRODUCTION

INTRODUCTION

The Gastrointestinal (GI) tract is a complex organ system with tissues that possesses distinct structural and functional characteristics. It performs the important task of processing the ingested food through digestion, absorption, expulsion of waste products and also protection against ingested microbes. It accomplishes these critical functions by the concerted action of the neuronal, muscular, immune and glandular tissue systems. The GI tract is highly innervated by local intrinsic neurons (the enteric nervous system) and the extrinsic sympathetic and parasympathetic nerves. This complex pattern of neural innervations control a variety of gut functions ranging from controlling the complex motor activity and propel the intraluminal content to the secretion of bioactive agents to facilitate digestion.

I. Structure of the Gastrointestinal Tract

The Gastrointestinal (GI) tract is a long tubular hollow structure with its wall divided into four distinct layers that pocesses characteristic morphological and functional property. The layering pattern of the gut from inside of the lumen to outside is as follows: mucosa, submucosa, muscularis externa, and serosa (Gabella 1987). The enteric nervous system (ENS) is consists of two ganglionated plexus within submucosa and muscularis externa. The ENS controls the GI function through intrinsic and extrinsic neural reflexes that are mediated through an array of messengers and neurotransmitters. The smooth muscle cells of the muscularis externa are aligned into two layers: an outer thin longitudinal muscle layer and an inner thick circular muscle layer. A large subpopulation of neurons, glial cells, fibroblasts, and interstitial cells of Cajal separates

the muscle layers (Kunze and Furness 1999). Smooth muscle cells are responsible for contractility of the GI tract.

I. 1. Enteric nervous system

The ENS consists of large number of nerve cell bodies that are found in the myenteric plexus located between the two smooth muscle layers and between the circular muscle layer and the submucosa (Gershon 1981). The ENS also contain glial cells that play an important role in providing support and nutrition to the neurons and are also involved in neurotransmission (Ruhl, Nasser et al. 2004). The enteric neural circuitry comprises of three functional classes of neurons that form multicomponent neural reflexes, viz motor neurons, interneurons and sensory neurons (Brookes 2001).

I. 1a. Motor neurons. Scientific investigation over the past decades has led to the identification of both excitatory and inhibitory motor neurons innervating the muscle layers in the gut. Commercial availability of selective receptor antagonist led to the identification of acetylcholine as a co-transmitter of excitatory motor neurons in addition to tachykinins (Grider 1989). Excitatory cholinergic transmission is mediated through muscarinic m_2 and m_3 receptors whereas; excitatory transmissions by tachykinin are mediated through NK_1 and NK_2 receptors (Lecci, Altamura et al. 2008). Similar to the excitatory motor neurons, the inhibitory neurons also encode for more than one transmitter (10) and include nitric oxide (NO), adenosine triphosphate (ATP), vasoactive intestinal peptide (VIP), and pituitary adenylyl cyclase-activating peptide (PACAP) (Harmar, Fahrenkrug et al. 2012).

Another subtype of motor neurons called the secretomotor neurons innervates the mucosal layer of the gut and stimulates the secretion from glandular tissue. The secretomotor

neurons have their cell bodies generally located in the sub-mucosal plexus and contain acetylcholine and VIP as co-transmitters (Carey, Cooke et al. 1985; Reed and Vanner 2007).

I. 1b. Interneurons. Physiological and pharmacological studies documented the existence of interneurons in the ENS with their cells bodies localized in the myenteric and submucosal plexus (Costa, Brookes et al. 1996). Based on structural evidences from guinea pig intestine, the interneurons are classified as ascending and descending interneurons (Neal and Bornstein 2008). Morphologically, the ascending reflex pathways represent a crosstalk between interneurons and excitatory motor neurons that is purely cholinergic acting through nicotinic receptors (Tonini and Costa 1990). However, the transmitters involved in the descending reflex pathways to relay information between the descending interneurons and the inhibitory motor neurons is clearly not elucidated, although, a possible involvement of ATP as a transmitter acting through purinergic P₂ receptors has been proposed (LePard, Messori et al. 1997; Xue, Farrugia et al. 1999).

I. 1c. Sensory neurons. Sensory neurons in the gut, acts as transducers to convey the information pertaining to its physical state and chemical milieu including villi movement, mucosal distortion, change in local distribution of blood flow, aberration in motility pattern and the chemical contents of the lumen, and integrate to the ENS (Furness, Jones et al. 2004). Morphologically, sensory neurons have been classified primarily as Dogiel type II neurons, which are characterized with multiple processes that form complex neuronal network establishing synaptic connections with other sensory neurons, interneurons, and motor neurons (Pompolo and Furness 1988). The sensory neurons exhibits well characterized electrophysiological properties, displaying broad action potential attributed to the presence of voltage gated sodium, calcium and potassium currents (Sanders 2000). Data gathered from recent

studies also implicated the involvement of sensory neurons in nociception due to their ability to trigger protective measures in response to noxious stimuli (Collins 1996; Lundgren 2002).

I. 1d. Neurotransmitters in the gastrointestinal tract

A variety of neurotransmitters that are expressed in the central nervous system are also found in the enteric nervous system of the gut. Some of the most important neurotransmitters involved in the gut function are briefly summarized below.

i) Acetylcholine. Acetylcholine (ACh) is one of the principal transmitters of the gut controlling important functions like motility and secretion (Tobin, Giglio et al. 2009). Acetylcholine is synthesized in neurons by the enzyme choline acetyl transferase (ChAT) and stored in vesicles where it can be released from nerve terminals upon appropriate stimulation (Schemann, Sann et al. 1993; Arvidsson, Riedl et al. 1997; Murthy, Zhou et al. 2003). Acetylcholine receptors can be broadly classified into two types nicotinic and muscarinic. Since the ENS contains more than 80% of cholinergic neurons, there must be other co-transmitters in the gut. The most commonly expressed co transmitter in the gut are neuropeptides (Furness 2006).

ii) Neuropeptides. The residual contractile effect in response to cholinergic antagonist paralleled the identification of the excitatory neuropeptide, tachykinins. The tachykinin family of neuropeptides constitutes substance P and neurokinin A (NKA) and neurokinin B (NKB) that are produced as preprotachykinins and undergo transcriptional and translational processing to form mature polypeptides. To date the neuropeptide family has been shown to interact with three receptors NK-1, NK-2 and NK-3 (Maggio 1988; Satake, Kawada et al. 2003). In contrast, the inhibitory component is mediated by vasoactive intestinal polypeptide (VIP). VIP belongs to a

structurally related family of peptides that includes secretin, PACAP and glucagon. On its release from nerve terminals, when stimulated, VIP acts on its membrane spanning G-protein coupled receptors and triggers the generation of the second messenger, cAMP. cAMP acts on its downstream effectors to mediate the relaxant effects of VIP (Holst, Fahrenkrug et al. 1987). Like other GI peptides, VIP is also secreted in its inactive precursor form that is processed to its active form through proteolytic cleavage (Grider 1993).

iii) Gaseous neurotransmitters. Gaseous transmitters are released on demand and diffuse across the cell membrane to act on specific intracellular targets. Nitric oxide (NO) is the most well studied gasotransmitter in the ENS, however recently carbon monoxide (CO) and hydrogen sulfide (H₂S) are gaining importance in regulating some of the GI functions (Kasperek, Linden et al. 2008).

The well defined characteristics of gaseous transmitters are as follows: a) they are gaseous molecules with very small atomic radii; b) they are freely diffusible across cellular membranes and do not activate cell surface receptors; c) they are synthesized *denovo* by enzymatic action and released on demand in a regulated way; d) they exhibit specific cellular functions at physiologically relevant concentrations; e) their cellular effects may or may not be dependent on the generation of second messengers; f) they should have very short half-life and efficient scavenging mechanisms to remove the transmitter following their action.

Nitric Oxide (NO). NO is generated as a gaseous product in the conversion of L-arginine to L-citruline by the enzymatic action of nitric oxide synthase (NOS). In the GI tract, NO is produced mainly by the action of constitutively expressed neuronal NOS (nNOS) and released by the inhibitory nitrergic neurons of the enteric nervous system (ENS) (Boehning and Snyder 2003). Following release NO diffuses through cell membrane and acts on its intracellular targets,

such as soluble guanylyl cyclase (sGC) to trigger the formation of cGMP. The cGMP formed then acts on several downstream effectors that collectively function to decrease the intracellular Ca^{2+} concentration and leads to hyperpolarization and smooth muscle relaxation (Koh, Sanders et al. 1996; Teng, Murthy et al. 1998). NO is the most prominent non-adrenergic, non-cholinergic neurotransmitter in the gut. Abnormalities in NO production have been correlated with many pathophysiological conditions such as achalasia, functional dyspepsia, delayed gastric emptying, diabetic gastroparesis and other GI dysmotility disorders (Shah and Kamath 2003).

Carbon Monoxide (CO). CO is produced as a catabolite of heme synthesis by the enzyme heme oxygenase (HO) along with other by-products such as biliverdin and iron. Release of CO by enteric neurons causes smooth muscle relaxation. Apart from stimulating sGC to produce cGMP, it also increases cAMP levels that lead to the activation of PKA (Xue, Farrugia et al. 2000; Wu and Wang 2005). The activation of second messenger-mediated kinases induces smooth muscle relaxation. Coexpression of HO and nNOS in myenteric neurons suggest a possible interaction between CO and NO. NOS potentiate CO mediated activation of sGC and enhance muscle relaxation. Defects in CO signaling are also associated with inflammatory and immune responses (Snyder, Jaffrey et al. 1998; Miller, Reed et al. 2001).

Hydrogen Sulfide (H_2S). H_2S has recently been identified as a gasotransmitter (Li, Bhatia et al. 2006). Endogenously, H_2S is produced by the action of two enzymes, cystathione γ -lyase (CSE) and cystathione β -synthase, on L-cysteine. The expression of these enzymes has been confirmed in ENS (Linden, Levitt et al. 2010). Following its release, H_2S is metabolized by several mechanisms including mitochondrial oxidation, cytosolic methylation, and glutathione-mediated oxidation. H_2S function of smooth muscle relaxation is primarily mediated by the activation of ATP-sensitive K^+ channels that lead to membrane hyperpolarization and subsequent

closure of voltage gated Ca^{2+} channels and relaxation (Ebrahimkhani, Mani et al. 2005). Studies have also shown that H_2S is involved in inducing chloride secretion in guinea pig and human colon (Schicho, Krueger et al. 2006; Pouokam and Diener 2012).

I. 2. Interstitial Cells of Cajal (ICCs)

The GI smooth muscle displays a distinct spontaneous electrical activity originating from interstitial cells of Cajal (ICCs). The smooth muscle cells are electrically coupled to ICCs and additionally integrate electrical inputs from ENS along with other endocrine and paracrine agents to regulate gut motility (JH. 1987; Sanders, Koh et al. 2006). The continuous discharge of electrical patterns by virtue of its pacemaker activity, ICCs enhances the open probability of voltage gated Ca^{2+} to induce sustained mode of contraction at different regions of the GI tract (Farrugia 1999; Bayguinov, Ward et al. 2007). This intrinsic rhythmic pattern of membrane potential in response to excitatory and inhibitory neural inputs is called slow waves. Basically, Slow waves function to shift the membrane potential of smooth muscle from a more negative potential to a more positive value, that helps to enhance the open probability of the voltage gated Ca^{2+} channels. The periodical influx of Ca^{2+} generating an inward current due to oscillatory opening of voltage gated Ca^{2+} channels helps to organize the excitation-contraction coupling of the muscle into a phasic pattern of contraction and relaxation (Morgan, Muir et al. 1981; Camborova, Hubka et al. 2003; Bayguinov, Ward et al. 2007).

Based on morphological and physiological evidences it has been suggested that interstitial cells of Cajal (ICC) are the pacemaker cells of the GI tract. Slow waves are initiated from ICCs and conducted to the smooth muscle cells as confirmed by electrical and optical recordings performed on ICCs and muscle cells simultaneously. In the absence of ICCs, muscle cells no longer shows rhythmicity and do not propagate slow waves. Experimental studies on

isolated and cultured ICCs have shown that ICCs express receptors for both excitatory and inhibitory transmitter (Ward, Burns et al. 1994; Huizinga, Thuneberg et al. 1995; Iino and Horiguchi 2006), the intracellular components and transmembrane ion channels necessary to generate spontaneous slow waves (Koh, Sanders et al. 1998). ICCs has also been shown to express different types of gap junction proteins including connexins, which help ICCs to form well ordered network of electrical coupling with the adjacent smooth muscle cells (Schwentner, Oswald et al. 2005). The cellular connectivity that ICC establishes with other ICCs and smooth muscle cells are crucial for its pacemaker activity.

I. 3. Gastrointestinal smooth muscle and its contractile apparatus

The smooth muscle in the gut produces contractile force to propel food along the length of the GI tract. Structurally, smooth muscle cells are about 200 to 300 μm in length and 5 to 15 μm wide. Unlike skeletal and cardiac muscles, smooth muscles are characterized by the lack of cross-striations that gives a smoother appearance in contrast to other types of muscle. Intracellular structural bodies such as dense bodies and contractile filaments populates in smooth muscle cells (Bitar 2003). Smooth muscle filaments can be categorized into three subtypes: thin actin filaments, thick myosin filaments and intermediate filaments. Actin is a 42-kDa protein that is ubiquitously expressed in its globular form called G-actin and polymerizes into double helical strands and forms filamentous actin or F-actin (Hodgkinson 2000; Rensen, Doevendans et al. 2007; Tang 2008). Thick filaments are hetero-multimeric proteins formed by the aggregation of six different types of polypeptides: 1 pair of myosin heavy chains (MHC) and 2 pairs of myosin light chains (MLCs) (Xu, Harder et al. 1996; Burgess, Yu et al. 2007). The MHCs forms a coiled structure that terminates in a globular head like structure surrounded by two pair of MLCs, a 20-

kDa regulatory light chain (MLC₂₀), and a 17-kDa essential light chain (ELC). The globular head contains a binding site for actin and actin-activated magnesium-adenosine triphosphatase (Mg-ATPase (Kamm and Stull 1989). Smooth muscle also contains thin filament associated proteins such as calponin, caldesmon, and smoothelin (Morgan and Gangopadhyay 2001).

I. 4. Signaling Mechanism Involved in Smooth Muscle Contraction

“Excitation-contraction coupling” in all muscle is initiated by increase in intracellular Ca²⁺ leading to changes in the activity of, or interactions of, actin and myosin. Actomyosin interaction is dependent on phosphorylation of MLC₂₀, which is both necessary and sufficient for smooth muscle contraction. Phosphorylation of MLC₂₀ leads to muscle contraction, whereas dephosphorylation leads to muscle relaxation. The levels of MLC₂₀ phosphorylation are regulated by MLC kinase (MLCK) and MLC phosphatase (MLCP) activity (Horowitz, Menice et al. 1996; Webb 2003; Huang, Zhou et al. 2005; Murthy 2006).

The signaling mechanisms for contraction in visceral smooth muscle is primarily mediated by G-protein coupled receptors (GPCRs). The GPCR apparatus consist of three tier membrane bound components: i) a seven transmembrane receptor protein, ii) a GTP-binding heterotrimeric G protein tethered to the receptor, and iii) membrane bound precursors that triggers the generation of one or more cellular signals that initiate cascades leading to activation of downstream effectors (Puetz, Lubomirov et al. 2009; Wright, Tripathi et al. 2012). Several subtypes of receptor were found to mediate the contractile response in smooth muscle cells and include receptors for acetylcholine, peptides (tachykinins, endothelin, motilin), amines (histamine, 5-hydroxytryptamine), pyrimidines/purines (UTP, ATP), and lipids (SIP, LPA)

(Morini, Kuemmerle et al. 1993; Hellstrom, Murthy et al. 1994; Murthy and Makhoulf 1998; Sarna 1999; Zhou and Murthy 2004).

I. 4.1. Regulation of myosin light chain kinase (MLCK).

In circular smooth muscle, activation of the receptor by the contractile agonist activates $G_{\alpha q}$ that leads to the activation of phosphoinositide (PI)-specific phospholipase C (PLC β 1). Stimulation of membrane bound PLC β 1 triggers the turnover of membrane embedded phosphatidylinositol 4,5-bisphosphate (PIP $_2$) that generates two important intracellular second messengers; the soluble calcium mobilizing agent IP $_3$ and the surface bound DAG. Soluble messenger, IP $_3$ binds to its high affinity receptors IP $_3$ R-I on the sarcoplasmic reticulum and drives the release of Ca $^{2+}$ into the intracellular milieu (Murthy, Grider et al. 1991). When Ca $^{2+}$ gets released, it associates with calmodulin to form an active Ca $^{2+}$ -calmodulin complex which then binds to and activates MLCK. The activation of MLCK causes phosphorylation of MLC $_{20}$, an obligatory step in smooth muscle contraction. Additionally, Ca $^{2+}$ influx through voltage-gated Ca $^{2+}$ channels also leads to activation of MLCK, phosphorylation of MLC $_{20}$ and muscle contraction (Makhoulf and Murthy 1997). The initial Ca $^{2+}$ surge responsible for initiating actomyosin interaction is very transient due to the operation of active Ca $^{2+}$ extrusion and intracellular Ca $^{2+}$ uptake mechanisms. These processes that causes downregulation of cytosolic Ca $^{2+}$ leads to uncoupling of Ca $^{2+}$ -calmodulin complex from MLCK, and results in the inhibition of MLCK activity and muscle contraction (Somlyo and Somlyo 1994; Murthy and Makhoulf 1997; Murthy 2006).

A number of studies have shown that MLCK itself is regulated by negative feedback mechanism through phosphorylation by specific protein kinases. CAMKII is one such kinase that

directly phosphorylates MLCK and inhibits its activity (Tansey, Luby-Phelps et al. 1994). Recently, another protein kinase called AMP kinase (AMPK) was also shown to phosphorylate MLCK at Ser⁸¹⁵ that causes significant reduction in its catalytic activity (Horman, Morel et al. 2008).

Although, the increase in Ca²⁺ and MLCK activity is transient, MLC₂₀ phosphorylation and muscle contraction are well maintained by Ca²⁺-independent mechanisms that exclusively involve processes that target inhibition of MLC phosphatase (Murthy and Makhoulf 1997; Ganitkevich, Hasse et al. 2002; Somlyo and Somlyo 2003).

I. 4.2. Regulation of myosin light chain phosphatase (MLCP)

In smooth muscle, regulated inhibition of MLCP is achieved by G-protein mediated activation of RhoA. Stimulation of two subsets of G-protein; G_q and G₁₃ in response to a contractile agonist leads to the activation of RhoA. RhoA belongs to a family of small G-proteins and in inactive state it is associated with GDP to form RhoA-GDP and localized in the cytoplasm. The GDP bound form of RhoA is stabilized by its interaction with another protein called guanine dissociation inhibitor (GDI). Signaling downstream of receptor activation stimulates RhoA, a process mediated by several Rho-specific guanine nucleotide exchange factors (RhoGEFs). When RhoGEFs is stimulated it promotes the release of GDP and exchange GTP in its place (Murthy and Makhoulf 1997; Somlyo and Somlyo 2003; Siehler 2009). RhoA-GTP is the activated form of RhoA, which then translocate from the cytoplasm and gets tethered to the inner leaflet of the plasma membrane and subsequently activates the smooth muscle specific isoform of Rho Kinase, Rho kinase II and also the membrane bound phospholipase D (PLD) (Murthy, Zhou et al. 2001).

When Rho kinase gets stimulated it act on MLCP to inhibit its activity. The MLCP holoenzyme comprised of three distinct component; a 110 to 130- kDa regulatory subunit known as the myosin phosphatase target subunit 1(MYPT1), a type 1 phosphatase (PP1c δ) of 37 kDa which is the catalytic subunit and, finally a 20 kDa subunit whose function is still not known (Hartshorne 1998). Rho kinase mediated phosphorylation of MYPT1 at Thr⁶⁹⁶ promotes uncoupling of the catalytic and regulatory subunits of MLCP that ultimately leads to inhibition of its catalytic activity (Fukata, Amano et al. 2001). Additionally, targeted phosphorylation of Thr⁸⁵³ located within the myosin-binding domain of MYPT1 by Rho kinase, promotes dissociation of MLCP from myosin and consequently decreases its efficiency to bind myosin (Wooldridge, MacDonald et al. 2004). Zipper interacting protein (ZIP) kinase, also known as the MYPT1-associated kinase, has a similar effect like Rho kinase on MYPT1 phosphorylation at Thr⁶⁹⁶ promoting its dissociation from the catalytic subunit and decreasing MLCP activity (MacDonald, Borman et al. 2001; Lincoln 2007).

As noted above, RhoA activation apart from stimulating Rho kinase also stimulates PLD activity. PLD hydrolyses phosphatidylcholine to generate phosphatidic acid. The latter gets subsequently dephosphorylated to yield DAG that causes prolonged activation of protein kinase C (PKC). Smooth muscle typically expresses the ϵ and δ isoforms of PKC that function to phosphorylate an endogenous MLCP inhibitor called CPI-17 (Murthy, Grider et al. 2000; Woodsome, Eto et al. 2001). PKC mediated phosphorylation of CPI-17 at Thr³⁸ greatly enhances its ability to inhibit MLCP and potentiates muscle contraction (Kitazawa, Eto et al. 2000). This dual role of RhoA to activate either Rho kinase or PKC is central to mediate the sustained phase of smooth muscle contraction.

Lack of inhibition of the sustained phase of smooth muscle contraction in the presence of inhibitors specifically targeted to Ca^{2+} /calmodulin dependent MLCK, suggests the involvement of Ca^{2+} -independent MLC Kinases during the sustained phase. Moreover, experimental evidences demonstrated that contraction in arterial smooth muscle cell by phosphatase inhibitor, microcystin-LR in Ca^{2+} free medium, is significantly abrogated by a non-specific kinase inhibitor. All of the above findings exclusively implicate an involvement of Ca^{2+} -independent MLCK to phosphorylated MLC_{20} and caused sustained contraction (MacDonald, Borman et al. 2001).

The most well characterized Ca^{2+} -independent kinases in smooth muscle cells are ZIP kinase and integrin-linked kinase (ILK). MLC_{20} phosphorylation at both Ser^{19} and Thr^{18} leading to contraction of permeabilized preparation of arterial smooth muscle in the presence of endogenously purified ZIP kinase and phosphatase inhibitor in Ca^{2+} free environment confirmed the involvement of ZIP kinase as a Ca^{2+} -independent kinase responsible for sustained contraction (Niir and Ikebe 2001; Muranyi, MacDonald et al. 2002). ZIP kinase is a serine/threonine kinase and a member of death-associated protein (DAP) kinase family. ZIP kinase has also been shown to colocalize with MYPT1 and phosphorylate the MLCP subunit at Thr^{696} in conjunction with Rho kinase (Borman, MacDonald et al. 2002). ILK is also a serine/threonine kinase that operates in a Ca^{2+} independent fashion and found to be associated with MLCP (Zhou, Das et al. 2003). ILK causes smooth muscle contraction either by directly phosphorylating MLC_{20} at Ser^{19} and Thr^{18} or by working in combination with CPI-17 to inhibit MLCP (Kiss, Muranyi et al. 2002; Zhou, Das et al. 2003; Huang, Mahavadi et al. 2006).

I. 5. Signaling Pathways Involved in Smooth Muscle Relaxation

Relaxation of contracted smooth muscle is mediated by generation of second messengers such as cAMP and cGMP, and activation of cAMP-dependent protein kinase (PKA) and cGMP-dependent protein kinase (PKG), respectively. PKA and PKG, in turn, act on various targets, and their action culminates in the reduction of intracellular Ca^{2+} and/or MLC_{20} phosphorylation, a prerequisite for muscle relaxation. The relaxant neurotransmitter in the gastrointestinal tract includes neuronal NO synthase (nNOS) and the peptide neurotransmitter, vasoactive intestinal peptide (VIP) and its homologue, pituitary adenylate cyclase-activating peptide (PACAP). Activation of soluble guanylyl cyclase by nitric oxide and G_s -coupled VPAC₂ receptors by VIP or PACAP result in the generation of cGMP and cAMP, respectively. Signaling cascades initiated downstream of cGMP and cAMP leads to efficient removal of Ca^{2+} transients causing inactivation of MLCK and subsequent MLC_{20} dephosphorylation. Muscle relaxation and MLC_{20} dephosphorylation can also be achieved by the removal of MLC phosphatase inhibition and successive augmentation of its dephosphorylating activity (Webb 2003; Murthy 2006).

I. 5.1. Regulation of Cyclic Nucleotide Levels

The titre of cAMP and cGMP in gastrointestinal smooth muscle is regulated by a balance between their synthesis by cyclases and degradation by phosphodiesterases (PDEs) (Turko, Francis et al. 1998). The cyclic AMP levels in smooth muscle are rapidly degraded by the action of cAMP specific PDEs, PDE3 and PDE4, whereas cGMP levels are regulated by cGMP-specific PDE5. PKA and PKG exert feedback regulation to fine-tune cAMP and cGMP levels via feedback regulation. PKA phosphorylates cAMP specific PDEs to stimulate their activity and subsequently promote cAMP degradation. On the other hand PKA mediated inhibitory

phosphorylation of adenylyl cyclase V/VI, the predominant isoforms in smooth muscle, also reduces the formation of cAMP. Similarly, cGMP levels are regulated via PKG-mediated stimulatory phosphorylation of PDE5 that promotes cGMP degradation, and inhibitory phosphorylation of sGC that attenuates cGMP generation (Francis, Turko et al. 2001; Murthy 2001; Murthy, Zhou et al. 2002).

I. 5.2. Molecular targets of PKA and PKG

In the initial phase of smooth muscle contraction, PKA and PKG induce relaxation primarily by terminating the Ca^{2+} transients and thereby decreasing the sensitivity of MLCK to Ca^{2+} . Both PKA and PKG in smooth muscle, inhibit IP_3 formation via downregulation of $\text{G}\alpha_q$ -dependent $\text{PLC}\beta 1$ activity. However the inhibition is not direct via inhibitory phosphorylation of $\text{G}\alpha_q$ and $\text{PLC}\beta 1$, but mediated by the combinatorial effect of PKA and PKG induced stimulatory phosphorylation of RGS4 and PKA induced phosphorylation of GRK2 that concertedly act to inhibit $\text{PLC}\beta 1$ activity (Huang, Zhou et al. 2007). Similarly, inhibitory phosphorylation of $\text{IP}_3\text{R}-1$ on sarcoplasmic reticulum by PKG but not PKA causes blockage of Ca^{2+} release into the cytoplasm (Murthy and Zhou 2003). PKG also phosphorylates sarco-endoplasmic reticulum $\text{Ca}^{2+}/\text{ATPase}$ (SERCA) and enhances Ca^{2+} uptake from the intracellular milieu into the sarcoplasmic store. Furthermore, PKG mediated stimulatory phosphorylation of plasmalemmal $\text{Ca}^{2+}/\text{ATPase}$ augments intracellular Ca^{2+} extrusion (Cornwell, Pryzwansky et al. 1991). Finally, both PKA and PKG inhibit the conductance through membrane bound Ca^{2+} channels and simultaneously activate K^+ channels to induce hyperpolarization (Koh, Sanders et al. 1996).

Relaxation can also be induced by PKA and PKG in a Ca^{2+} independent manner during the sustained phase of contraction. The inhibition of the catalytic activity of MLCP through

RhoA pathway is released by PKA and PKG mediated phosphorylation of the molecular targets upstream as well as downstream of RhoA. The upstream targets of RhoA by the cyclic nucleotides include the $G_{\alpha 13}$, p115 RhoGEF and RhoA and the downstream targets include Rho kinase, and MYPT1. Both PKA and PKG can phosphorylate RhoA at Ser¹⁸⁸ and promote its translocation from the plasma membrane to the cytosol. The movement of RhoA from membrane to cytosol strictly inhibits Rho kinase activity by impeding its physical interaction with membrane-bound molecular targets. Therefore, the inhibitory effect of Rho kinase on MLCP via MYPT1 phosphorylation is withdrawn. Additionally, the inhibitory effect of CPI-17 on MLCP activity is released via inhibition of Rho kinase/PLD activity and PKC mediated phosphorylation of CPI-17 (an endogenous MLCP inhibitor) at Thr³⁸ (Sauzeau, Le Jeune et al. 2000; Murthy, Zhou et al. 2003; Murthy 2006). Lastly, relaxation mediated via RhoA independent pathway is also enhanced by stimulatory phosphorylation and activation of telokin, an endogenous MLCP activator (MacDonald, Walker et al. 2000). All of the above effects of cAMP/PKA and cGMP/PKG converge to produce enhanced activity of MLCP and accelerated dephosphorylation of MLC₂₀, a hallmark for smooth muscle relaxation.

I. 6. Receptor Desensitization and Internalization

Over the last two decades multiple molecular mechanisms has been proposed to explain the regulation of GPCR signaling. Primarily, following activation of the GPCRs by their cognate ligands, specific signaling mechanism gets triggered to produce an appropriate cellular response. The intensity of the signaling cascade is regulated by reducing the efficacy of the GPCRs to transmit signal through a process called desensitization, or by the subsequent removal of the activated receptors from the surface to blunt the signaling response through receptor

internalization (Drake, Shenoy et al. 2006). Desensitization refers to the process that leads to reduce ability of the receptor to get stimulated as a consequence of prior enhanced activation (Hausdorff, Caron et al. 1990).

The term desensitization has traditionally been used to describe events that cause the uncoupling of the activated receptors from heterotrimeric G-proteins. In contrast, sensitization refers to the process that causes increased ability of the receptor to get stimulated due to low activation (Gainetdinov, Premont et al. 2004). GPCRs exhibit desensitization in response to its activating ligands to regulate the signaling intensity and promote signaling termination. GPCR mediated signaling is regulated at the receptor level majorly by two mechanisms: i) by modulating signaling efficacy of the receptors and ii) by upregulating or downregulating the number of cell surface receptors. Desensitization process can be further categorized into two types: homologous desensitization that is dependent on ligand-receptor interaction and the heterologous desensitization that is independent of ligand-receptor interaction (Bohm, Grady et al. 1997).

I. 6.1. Molecular mechanism of homologous desensitization

Ligand bound GPCRs not only activates G-proteins to initiate specific signaling cascades, but also the interacting ligand-receptor complex forms a substrate for protein phosphorylation (Delom and Fessart 2011). Following receptor activation by the ligand, G protein coupled receptor kinase (GRK) gets recruited to the cytoplasmic domain of the activated receptor and phosphorylate the receptor at specific sites (Shenoy and Lefkowitz 2003). The GRK family of kinases comprised of seven members that has been categorized into three subfamilies, viz. GRK 1/7, GRK 2/3 and GRK 4/5/6 on the basis of significant overlap of the amino acid sequence and

structural homology (Pitcher, Freedman et al. 1998; Willets, Challiss et al. 2003). GRK 2, 3, 5 and 6 were found to be widely expressed and has been implicated in regulating most of the GPCR signaling. The other members of the GRK family GRK 1/7 are exclusively involved in signal transduction in visual pathways, whereas GRK4 is only expressed in the testes (Gainetdinov, Premont et al. 2000; Penela, Murga et al. 2006).

GRK mediated phosphorylation of the activated receptor is a prelude to the binding of a scaffolding protein called β -arrestin. The driving force of arrestin recruitment comprises of both the GRK phosphorylation sites on the receptors and the active ligand-receptor configuration (Perry and Lefkowitz 2002). The primary role of arrestin interacting with the activated receptor is to dampen the firing of the ligand-receptor complex. The inhibition in signal transmission by the ligand-receptor complex is achieved by restricting the G_{α} subunit from exchanging GTP for GDP. Locking the G_{α} subunit in the GDP conformation prevents the transmission of cellular signal to the downstream effectors (Luttrell and Lefkowitz 2002). Among the most well characterized arrestins are the nonvisual arrestins called the β arrestins viz. β arrestins-1 and β arrestins-2. They are widely expressed in a variety of tissue and play an important role in regulating many GPCR signaling machineries.

I. 6.2. Molecular mechanisms of heterologous desensitization

Heterologous desensitization involves attenuation of the signaling downstream of the receptor and precludes physical interaction between the receptor and the ligand (Chuang, Iacovelli et al. 1996). An efficient way of receptor uncoupling from the heterotrimeric G-protein involves covalent modifications in the structure as imposed by receptor phosphorylation. The second messenger dependent kinases such as protein kinase A (PKA) and protein kinase C

(PKC) catalyzes transfer of γ -phosphate group of ATP to serine and threonine residues of its target proteins (Pierce, Premont et al. 2002). PKA and PKC are activated downstream of GPCR signaling and causes generation of second messengers such as cAMP, increase in intracellular Ca^{2+} and diacylglycerol. The second messengers generated activate the downstream kinases and transduce signal by stimulating them through phosphorylation (Ferguson 2001). Apart from phosphorylating their target effectors, these kinases by virtue of feedback regulation, phosphorylates the receptors. Second messenger activated protein kinase mediated phosphorylation of the receptors leads to a marked loss of the receptor function. Unlike GRKs that selectively phosphorylated agonist bound receptors, second messenger dependent protein kinases in contrast, randomly phosphorylates receptors irrespective of the presence or absence of the agonist. Moreover, the sites of receptor phosphorylation by these kinases are different from those of GRKs (Lefkowitz, Hausdorff et al. 1990). In addition to negatively regulating the receptors through phosphorylation, PKA and PKC phosphorylates several other targets such as adenylyl cyclase, phospholipase C that collectively contribute to the desensitization process (Hamm and Gilchrist 1996).

I. 6.3. Deactivation of G-protein by RGS proteins

RGS (regulators of G protein signaling) proteins form a distinct class of regulatory proteins containing a conserved 120 amino acid sequence called RGS domain that act as GTPase activating protein (GAPs). The RGS domain binds specifically to the activated G_α subunit and increases the rate of GTP hydrolysis and stabilizing the GDP-bound conformation of the G-protein. Thus by enhancing the intrinsic GTPase activity of the G-protein, they dampen the intensity of signaling pathways (Berman and Gilman 1998; Neubig 2002). Recently it has also

been demonstrated that RGS proteins can directly interact with the receptors and modulate their activity (Snow, Hall et al. 1998).

I. 7. GPCR internalization

Agonist-activated receptor internalization into intracellular pockets is an important aspect of regulation of GPCR activity. GRK phosphorylation of the activated receptors generates high affinity binding sites for β -arrestins (Delom and Fessart 2011). Binding of coat proteins clathrin follows binding of β -arrestins to the receptor, which exhibits high affinity to β -arrestins and interacts through their carboxy terminal region. Overall, the process involves translocation of β -arrestins from the cytoplasm to the plasma membrane and bind to the receptor at the site of GRK phosphorylation to mediate the process of receptor internalization via both clathrin-dependent and clathrin-independent caveolar pathway (Traub 2003). The model pathway for internalization is exemplified by β 1-adrenergic receptors internalization, which is selectively mediated via clathrin-dependent pathway when phosphorylated by GRK, and by clathrin-independent/caveolar-dependent pathway when phosphorylated by PKA (Rapacciuolo, Suvarna et al. 2003). Additionally, lipid modification such as palmitoylation/depalmitoylation of receptors has also been shown to play a role in targeting the receptors to lipid rafts for internalization (Papoucheva, Dumuis et al. 2004).

I. 8. Regulation of GPCR Signaling by Scaffolding Proteins

Higher degree of specificity in the molecular interaction among multitude of signaling proteins forms the basic tenet of the signal transduction process. It is of primary importance to study the mechanisms involved in bringing the molecular partners of a signaling cascade in close

proximity for accurate and efficient signaling events. Scaffolding proteins forms a special class of regulatory proteins that essentially act as molecular glue to gather signaling molecules in close proximity that accounts for the precise and efficient signaling mechanisms. Scaffolding proteins provides the basic platform that helps in subcellular colocalization of signaling molecules by associating with protein components of a signaling cascade and enhancing protein-protein interaction for an efficient transmission of cellular signals (Faux and Scott 1996; Wrana and Pawson 1997). The hallmark of interaction of a scaffolding protein is defined by the presence of a unique signature motif that helps them to interact and bind with their target proteins. The function of a scaffolding protein to facilitate signal transduction may not necessarily be restricted to mere tethering of signaling molecules to promote the assembly of a signaling complex, but also to modulate/regulate the activity of its protein partners. The major function ascribed to scaffolding proteins can be broadly classified into two broad areas- firstly, isolating signaling molecules of a cascade into a pre-signaling complex and secondly, regulating the activation and or inhibition of components of a signaling pathway (Good, Zalatan et al. 2011). Some of the important scaffolding proteins involved in the regulation of GPCR signaling are summarized below with particular emphasis on caveolin proteins.

I. 8.1. A-Kinase Anchoring Proteins (AKAPs). AKAPs constitute the first family of proteins that was recognized with a scaffolding function. AKAP associate with its main host protein, PKA along with several other proteins of a signaling pathway and co-localizes them in a specific sub-cellular location (Michel and Scott 2002; Pidoux and Tasken 2010). The role of AKAP regulation of signaling pathways is exemplified by the activation of G_s -coupled β -adrenergic receptors (β AR) by its specific ligand epinephrine, in response to stress to regulate

cardiovascular function. To date many AKAP isoforms has been reported in a range of species, including yeast, fruitflies, mice and humans. All AKAPs share some common characteristic features such as a PKA-binding motif, a localization signal that helps to tether the protein to the plasma membrane and the ability to form presignaling protein complex with other components of a signaling pathway (Diviani and Scott 2001; Gold, Lygren et al. 2006; Pidoux and Tasken 2010).

I. 8.2. PDZ-domain containing scaffolding proteins. PDZ domains are protein-protein interaction domains found in several proteins. The name is derived from the initial letters of the PSD-95, Dlg, and a ZO-1 protein that contains the sequence repeats from which the initial discovery was made. Generally, the interaction between the PDZ domain and its target protein though the C-terminal region is constitutive, however, in some instances the interaction also entails agonist-dependent receptors activation (Gomperts 1996; Ranganathan and Ross 1997; Feng and Zhang 2009). Conclusive evidence exists to support that β 2-adrenergic receptors interact with proteins containing PDZ-domain that acts as scaffolds to link the receptor to its downstream effectors (Hall and Lefkowitz 2002; Valentine and Haggie 2011). Interestingly, GRK mediated phosphorylation of β 2-adrenergic receptors completely disrupt the association of the receptor to its PDZ-containing binding partners that clearly shows that GPCRs interaction with PDZ-domain containing proteins is critically dependent on GRK phosphorylation. Similarly, phosphorylation of inwardly rectifying K^+ channels by PKA also disrupts its interaction with other PDZ-domain harboring proteins (Cohen, Brenman et al. 1996; Cao, Deacon et al. 1999; Fanning and Anderson 1999; Hu, Chen et al. 2002).

I. 8.3. Tetratricopeptide repeat (TPR)-bearing scaffolding proteins. This subclass of scaffolding proteins have been found to interact with different subtypes of G_α subunits including, $G_{\alpha s}$ and $G_{\alpha q}$. The TPR bearing scaffolding proteins been shown to preferentially interact with the active conformation of HA-Ras in the absence of the G-protein, $G_{\alpha 16}$. Although, the physiological relevance of this interaction has not been explored in detail (Marty, Browning et al. 2003; Andreeva, Kutuzov et al. 2007).

I. 8.4. β -arrestins. The aforementioned interactions of GPCR with scaffolding proteins strictly entail the presence of specialized sequence motifs embedded either in the receptor or present in the scaffolding proteins. The association of GPCRs with β -arrestins exhibits a variation to the rule of specific GPCR/scaffold interaction. The family of β -arrestins exist in two isoforms: β -arrestins1 and β -arrestins2 both are exclusively involved in the desensitization process of the β -AR. However, recent studies has revealed that β -arrestins can associate with a broad variety of GPCRs. G-protein receptor kinase (GRKs) mediated phosphorylation of the activated receptors, promotes the receptor association with β -arrestins and causes uncoupling of the receptor from the G-protein (Luttrell and Lefkowitz 2002; Lefkowitz and Whalen 2004). Several proteins involved in endocytotic pathway such as clathrin are also known to interact with β -arrestins. The interactions of β -arrestins with the endocytotic proteins facilitate ligand induced receptor internalization. This qualifies β -arrestins as scaffolding protein linking GPCRs with the endocytotic machinery to promote internalization. Additionally, β -arrestins are also known to interact with tyrosine kinase Src and the interaction is important for activation of Src kinase to mediate other signaling events. Thus, β -arrestins are not only proteins merely involved in GPCR internalization but can also function as a protein scaffold to simultaneously stimulate parallel

signaling processes (Luttrell, Ferguson et al. 1999; Imamura, Huang et al. 2001; Ma and Pei 2007).

I. 8.5. Caveolin proteins. Caveolae are 50 to 100 nm omega shaped, cell surface membrane invaginations found in many cell types including vascular endothelial cells, muscle cells, adipocytes and fibroblasts (Parton and Simons 2007; Volonte, McTiernan et al. 2008). Previous studies demonstrated myriad signaling molecules populate in caveolar pits including different type of receptors such as GPCRs coupled to heterotrimeric and monomeric G-proteins, receptor tyrosine kinases and other growth factor receptors (Wu, Butz et al. 1997; Okamoto, Schlegel et al. 1998; Chini and Parenti 2004). Caveolae have been implicated to regulate important cellular processes like endocytosis, transcytosis, calcium signaling and several other signal transduction events (Minshall, Sessa et al. 2003; Cohen, Hnasko et al. 2004). Caveolae forms a distinct subset of lipid rafts that are formed by the dynamic aggregation of cholesterol, glycosphingolipids and sphingomyelin (Ortegren, Karlsson et al. 2004). They are also highly enriched with several resident proteins of the plasma membrane such as glycosphosphatidylinositol (GPI-anchored) and other phospholipids (Lisanti, Tang et al. 1993; Lisanti, Scherer et al. 1994).

The caveolae coat proteins, caveolins serves as markers for identification of the caveolar domains and are critical for caveolae function. Caveolins are approximately 22-kDa proteins consisting of 178 amino acids with multiple acetylation and phosphorylation sites. Three isoforms of caveolin proteins: caveolin-1, caveolin-2 and caveolin-3, has been identified to date (Williams and Lisanti 2004; Chidlow and Sessa 2010). Caveolin-1 also known as vesicular integral membrane protein (VIP) was the first member of the caveolin gene family to be identified (Razani, Woodman et al. 2002; Parton 2003). It displays a wide range of expression

pattern in many tissue types including non-muscle and smooth muscle tissues. In smooth muscle, the first member of the caveolin family, caveolin-1 alone drives the formation of caveolae and forms the major structural components that preferentially interacts with different signaling molecules and regulate their function. In contrast, caveolin-2 is nonessential for caveolae formation and colocalizes with caveolin-1 for its stable expression. Although, caveolin-2 is not essential for caveolae formation, however co-expression and hetero-oligomerization of caveolin-1 and 2 forms a more deeper and abundant caveolae, indicating a modulatory role of caveolin-2 in caveolae formation. The last dominant isoform of the caveolin family, caveolin-3 is a muscle specific isoform and share high structural and functional similarities with caveolin-1. Unlike caveolin-2, caveolin-3 can form caveolar microdomain independent of caveolin-1 (Tang, Scherer et al. 1996; Scherer, Lewis et al. 1997; Williams and Lisanti 2004; Mercier, Jasmin et al. 2009).

Sequence analysis studies have shown that caveolins interact with their binding partners through a short stretch of amino acids known as the caveolin scaffolding domain (CSD) embedded in the cytosolic N-terminal region spanning from amino acids 82 to 101 (Couet, Li et al. 1997; Epand, Sayer et al. 2005). Additionally, the interactions are also guided by a defined sequence of amino acids called the caveolin binding motifs (CBM) integrated within the binding partners. The most well defined CBM that has been identified contains a short tandem protein sequence with aromatic amino acids inserted in a specific pattern (Razani, Woodman et al. 2002; Collins, Davis et al. 2012).

I. 8.5a. Regulation of endothelial nitric oxide synthase by caveolin. The interaction of caveolin proteins with endothelial nitric oxide synthase (eNOS) has been explored extensively (Garcia-Cardena, Fan et al. 1996). Studies demonstrated that interaction of eNOS with CSD of

the caveolin protein leads to inhibition of its enzymatic activity. Both caveolin-1 and -3 were shown responsible for suppressing the catalytic activity of eNOS. A number of studies also supported the hypothesis that decreases in caveolin expression upregulates eNOS activity under basal condition (Venema, Ju et al. 1997; Feron and Kelly 2001; Rath, Dessy et al. 2009).

I. 8.5b. Regulation of cAMP/cGMP signaling by caveolin. The scaffolding domain of caveolin-1 and -3 also interacts with specific isoforms of adenylyl cyclase (AC) and inhibit their activity (Toya, Schwencke et al. 1998; Willoughby and Cooper 2007). Consequently, disruption of caveolae or downregulation of caveolin proteins significantly promotes adenylyl cyclase activity (Yamamoto, Okumura et al. 1999; Head, Patel et al. 2006). Additionally, cyclic nucleotide phosphodiesterases (PDE), the enzyme responsible for hydrolyzing cAMP and cGMP has also been reported to localize in caveolar microdomains (Abrahamsen, Baillie et al. 2004; Baillie, Scott et al. 2005). Previous studies have shown that PDE3B isoform is compartmentalized in caveolae and its expression was decreased considerably in caveolin-1 knockout mice (Nilsson, Ahmad et al. 2006). Similar association of PDE5 isoform with caveolae was reported and its expression decreased with targeted suppression of caveolin proteins with siRNA techniques and also in caveolin-1 knockout mice (Murray F 2006). Although, preliminary studies claims that caveolin-1 inhibits the catalytic activity of protein kinase A (PKA), however the localization of PKA in caveolae is not very well defined and remains inconclusive (Razani, Rubin et al. 1999; Razani and Lisanti 2001).

I. 8.5c. Regulation of receptor tyrosine kinases by caveolins. Receptor tyrosine kinases (RTK), another major class of transmembrane receptor was also reportedly associated with or

directly localized in caveolae (Couet, Sargiacomo et al. 1997; Pike 2005). Studies from several groups have shown that caveolin proteins regulate the MAP kinase cascade components, which forms the downstream effectors of RTK. Studies involving overexpression of caveolin isoforms leads to a marked downregulation of the signaling through the MAP kinase pathway (Engelman, Chu et al. 1998). In airway and vascular smooth muscle the expression of caveolin-1 was markedly reduced in proliferating muscle cells. Consistent with the antiproliferative role of caveolins, expression of receptors for growth factors such as EGF and PDGF, and constituent of growth factor signaling cascades such as Src, Shc, Grb2 and Ras are localized in caveolae. The selective interaction between the caveolin proteins with the members of the MAP kinase cascade significantly suppresses their activity (Gosens, Stelmack et al. 2006; Gosens, Stelmack et al. 2011). This shows a clear regulation of growth factor signaling by caveolae and its defining protein caveolins.

I. 8.5d. Regulation of ion channels by caveolins. Ion channels and transporters play an important role in a variety of cellular functions that contribute in maintaining the membrane potential and cellular excitability to the release of neurotransmitters. Many groups have reported the potential localization of different kinds of ion channels such as Ca^{2+} , K^{+} , Na^{+} , and Cl^{-} in caveolar pits (Taggart 2001; Isshiki and Anderson 2003; Bergdahl and Sward 2004). Subcellular localization of these transmembrane proteins in rafts and caveolar microdomain are critical for their modulation by different cellular signals. The active components of the intracellular calcium handling machinery such as Ca^{2+} -ATPase, IP_3 receptor, voltage gated calcium channels, calmodulin, calcium pumps and transient receptor potential (TRP) channels, are preferentially harbored in cholesterol rich plasma membrane domains including caveolae in many cell types

(Fujimoto, Miyawaki et al. 1995; Schnitzer, Oh et al. 1995; Darby, Kwan et al. 2000; Lohn, Furstenau et al. 2000; Wang, Ye et al. 2005). Subcellular localization of these proteins in caveolar domains clearly indicates that caveolae and caveolin proteins conclusively participate in the process of intracellular calcium signaling.

I. 8.5e. Regulation of endocytosis by caveolin proteins. Caveolae has been proposed to mediate the process of transcytosis in order to shuttle macromolecules across endothelial membrane. This process of transcytosis involves GTP-dependent, dynamin-mediated internalization of caveolar microdomains from the plasma membrane (Oh, McIntosh et al. 1998; Shajahan, Timblin et al. 2004; Predescu, Predescu et al. 2007). Internalization of caveolae is also triggered by other signals that include actin polymerization, presence of cholesterol, the presence of membrane protruding GMI ganglioside and phosphorylation through kinases (Schnitzer, Liu et al. 1995; Mukherjee, Tessema et al. 2006). The role of tyrosine kinase Src is remarkably well elucidated to understand its role in the molecular mechanisms of caveolar endocytosis. Downregulation of Src using small interfering RNA has resulted in enhanced accumulation of caveolar structure at the cellular membrane. Following endocytosis, the caveolar structure is maintained as a stable unit that ultimately fuses with other intracellular compartments such as caveosome in a RAB5 dependent manner. These intracellular caveolar units can later be recycled back to the plasma membrane to restore the caveolar topology on the membrane (Pelkmans, Burli et al. 2004; Shajahan, Tiruppathi et al. 2004; Parton and Simons 2007).

I. 9. Regulation of smooth muscle function by caveolins

Studies from several laboratories have reported a very crucial role of caveolae to organise and regulate smooth muscle function by modulating cellular processes such as contraction, and growth and proliferation. Although, caveolin knockout mice are viable, however, they are more prone to develop pathophysiological complications such as cardiomyopathy, enhanced eNOS activity and severe pulmonary dysfunction (Drab, Verkade et al. 2001; Zhao, Liu et al. 2002; Cohen, Hnasko et al. 2004; Chidlow and Sessa 2010). All these have drawn scientific attention to investigate any modulatory effect of caveolae and caveolins in smooth muscle function. Several studies demonstrated caveolar colocalization of ion channels and signaling molecules involved in contraction (Maguy, Hebert et al. 2006; Balijepalli and Kamp 2008). Recently, caveolae and its coat proteins have been shown to play an important role in 5-HT_{2A} and endothelin-1 (ET-1) receptor mediated smooth muscle contraction and disruption of caveolae severely compromised smooth muscle contractility (Cristofaro, Peters et al. 2007). The involvement of caveolae in regulation of L-type Ca²⁺ channel is controversial. Darby et al provided evidence of caveolar enrichment of L-type Ca²⁺ channels and Ca²⁺ binding proteins and their direct modulation by caveolae to control smooth muscle contractility (Darby, Kwan et al. 2000). However, Lohan et al reported that caveolae downregulation has no effect on conductance through L-type Ca²⁺ channels. Caveolae has also been implicated to mediated Ca²⁺ sensitisation process through its interaction with Rho kinase and several isoforms of PKC and regulate arterial smooth muscle contraction. Targeted erosion of caveolar pits from arterial smooth muscle cells caused significant impairment in muscarinic m2 and m3 receptor mediated airway smooth muscle contraction (Schlenz, Kummer et al. 2010). Moreover, response of ileum longitudinal muscle to endothelin-1, but not to carbachol or serotonin was reduced in caveolin-1 KO mice, whereas the

response of femoral arterial muscle to α 1-adrenergic agonist was increased (Shakirova, Bonnevier et al. 2006). Thus, it appears that regulation of smooth muscle contraction by caveolins is both tissue- and receptor-specific.

I. 10. Novelty and Significance

Caveolae provide an essential platform for signal transduction by G protein-coupled receptors in vascular and airway smooth muscle contractile function by integrating the contractile signals and intracellular effector pathways. Recent studies using caveolin knockout animals demonstrated that caveolae and caveolin-1 play an important role in several diseases phenotypes such as diabetes, athresclerosis, cardiac hypertrophy, pulmonary hypertension and bladder dysfunction. Studies in vascular and airway muscle also demonstrated that the expression of caveolin-1 was significantly reduced in proliferating muscle cells compared to contractile phenotype. Caveolins, therefore, are also important for the maintenance of smooth muscle contractile phenotype. Gastrointestinal motility depends on the integrated function of enteric neurons, interstitial cells of Cajal and smooth muscle cells. Studies examining the involvement of caveolae and caveolin-1 in the regulation of GI motility are limited and, often, restricted to the intact tissue or organ. An obvious conclusion from these studies is the importance of caveolae and caveolins in the regulation of contractile function. Undoubtedly, the next step in the process of understanding the mechanism by which caveolae and caveolins regulate muscle contraction is to understand the regulation of signal transduction pathways activated by contractile neurotransmitters in the smooth muscle. The signal transduction pathways that regulate muscle contraction short-term also regulate smooth muscle phenotype long-term. In this study we examined the *hypothesis that caveolae and caveolin-1 provide a critical regulatory environment for excitation-contraction and excitation-transcription coupling in the smooth muscle cells*. To test this hypothesis we investigated the role of caveolae in the regulation of gastric smooth muscle function by the main excitatory neurotransmitter, acetylcholine mimetic, carbachol using freshly dispersed muscle cells devoid of neurons and

ICC. The role of caveolae and caveolin-1 in m2 and m3 receptor signaling was examined using pharmacological, biochemical, molecular and genetic approaches. Our study provides important biochemical basis by which caveolin-1 can regulate the integration of extracellular contractile stimuli and the downstream intracellular effectors in smooth muscle. For perspective, as demonstrated in the present study, caveolin-1 represents an important scaffolding protein in orchestrating G protein-coupled receptor signaling to have a dual role in pro- excitation-contraction and excitation-transcription, and anti-proliferation in gastric smooth muscle. These results may also help to establish caveolin-1 as a therapeutic target in the treatment of smooth muscle complications in GI motility disorders.

CHAPTER: II MATERIALS AND METHODS

II. 1. Materials

[¹²⁵I]cAMP, [γ -³²]ATP, [³H]myo-inositol, [³H]*Scopolamine* were obtained from PerkinElmer Life Sciences (Boston, MA); methoctramine (muscarinic m2 receptor antagonist) Sigma-Aldrich St. Louis, MO; 4-DAMP (muscarinic m3 receptor antagonist) Sigma-Aldrich, (St. Louis, MO); Y27632 (Rho kinase inhibitor) Calbiochem, (La Jolla, CA); U73122 (PLC inhibitor) Enzo Life Sciences, Inc., NY; PP2 (Src inhibitor) Enzo Life Sciences, Inc., NY; Antibodies: Caveolin-1, caveolin-3, phospho-MYPTI, phospho-MLC₂₀, α -actin, γ -actin, caldesmone, β -actin, Rho kinase II, ZIP kinase, muscarinic m2 receptor, muscarinic m3 receptor, SRF, myocardin, phospho EGF receptor, ERK1/2 were obtained from Santa Cruz biotechnology, Santa Cruz, CA; RNAqueousTM kit was obtained from Ambion, Austin, TX; LipofectamineTM 2000 transfection reagent, SuperScriptTM II Reverse Transcriptase kit, DH5- α competent cells, were obtained from Invitrogen, Carlsbad, CA; Restriction enzymes were obtained from New England Biolabs (Ipswich, MA); QIAprep^R Spin Miniprep Kit was obtained from QIAGEN Sciences, Maryland; PCR reagents were obtained from Applied Biosystems, Roche. Caveolin-1 SiRNA was obtained from BD Biosciences, San Jose, CA. pSIREN-DNR-DsRed vector was obtained from Clontech Laboratories, Inc. CA; EGF was obtained from BD Biosciences, San Jose, CA. Collagenase CLS type II and soybean trypsin inhibitor for cell isolation were obtained from Worthington, Freehold, NJ; Western blotting materials, Dowex AG-1 X 8 resin (100-200 mesh in formate form), chromatography materials and protein assay kit, Tris-HCl ready made gels were all obtained from Bio-Rad Laboratories, Hercules, CA; Dulbecco's modified Eagle's medium for primary cell culture was obtained from Fisher Scientific. All other chemicals were obtained from Sigma, St. Louis, MO.

Animals

New Zealand white rabbits (weight: 3-4 lbs) were purchased from RSI Biotechnology, Clemmons, NC and killed by injection of euthasol (100 mg/Kg), and caveolin-1 knockout and wild type mice (B6129SF2/J) were obtained from the Jackson Laboratory (Bar Harbor, ME) and killed by suffocation with CO₂. All the procedure was followed as approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University. The animals were housed in the animal facility maintained by the Division of Animal Resources, Virginia Commonwealth University. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Table 1: Primers for qRT-PCR

Target	Forward Primer 5'-3'	Reverse Primer 5'-3'
m1 receptor	GGTGGCAGCAGCAGCAGCTCA	GCCTCTTGACTGTATTTGGGGAGC
m2 receptor	GTGACTGAAACCTGTGTT	GGAGGCTTCTTTTTGGCAGGCTG
m3 receptor	AGCTCCATCCTCAACTC	GGTTTTGGGTATGCAGCTGTCAC
m4 receptor	TGAGTCCAGCTCAGGCAGTGCC	GTTCCGATACTGGCACAGCA
m5 receptor	GAAAGGAATCAGGCCTCCTGG	CCACCAATCGGAACTTATAGGC
Caveolin-1	GACGCGCACACCAAGGAG	GTCACAGTGAAGGTGGTG
Caveolin-3	GTCTCCAAGTACTGGTGC	GGGTTGCAGAAGGTGCGG
γ-actin	ATCAGGCCCGCGACAACCTCGT	GCCGCCAGTTGTGCTCTAAAAGGT
Caldesmon	CAGAAGGGAAGTCGGTAAATGAAA	GGGCAGCTGTCTGAAGTTTATCTTCTT
β-actin	CCCTCCATCGTGCACCGCAA	CTCGTCTCGTTTCTGCGCCGT
GAPDH	GCCTGGAGAAAGCTGCTAAGTATG	CCTCGGATGCCTGCTTCA
Myocardin	CGGATTCTGAAGCTGTTGTCTT	AAACCAGGCCCCCTCCC
SRF	TCTCAGGCACCATCCACCAT	CCCAGCTTGCTGTCCTATCAC
SM-MHC	TGGACACCATGTCAGGGAAA	ATGGACACAAGTGCTAAGCAGTCT

II. 1. METHODS

II. 2. 1. Tissue collection and processing

Rabbits were sacrificed by injecting euthasol at a concentration of 100mg/kg of body weight into the ear vein. The stomach was rapidly isolated and removed of its content, washed and placed in pre-chilled smooth muscle buffer (pH 7.4) with the following composition: 120 mM NaCl, 4 mM KCl, 2.6 mM KH_2PO_4 , 2 mM CaCl_2 , 0.6 mM MgCl_2 , 25 mM HEPES (N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid), 14 mM glucose, and 2.1% essential amino mixture.

II. 2.2. Preparation of dispersed gastric smooth muscle cells

The stomach was separated into thin distal fundus and the thicker posterior antrum. Smooth muscle cells were isolated by sequential enzymatic collagenase digestion from the circular muscle layer followed by filtration and collection through Nitex mesh and centrifugation as described previously (Bitar and Makhoulf 1982; Murthy, Zhou et al. 2001). After removal of the mucous layer by sharp dissection from both regions, the fundus was chopped and minced thoroughly with a pair of sharp scissors and the antrum was sliced into thin pieces using a Stadie-Riggs tissue slicer. Following tissue processing, they were incubated separately in a solution of smooth muscle buffer containing 0.1% collagenase (300U/ml) and 0.001% soybean trypsin inhibitor (w/v) for 30 min at 31⁰C in a water bath with a constant supply of 100% oxygen. The partially digested tissue was washed twice with collagenase free smooth muscle buffer and the smooth muscle cells were allowed to disperse spontaneously for approximately 30 min in the enzyme free medium. At the end of digestion and dispersion the cells were harvested by filtration through 500 μm Nitex mesh and centrifuged twice at 350 g for 10 min to eliminate broken cells

and cellular organelles. With an estimated 95% of the cells excluded trypan blue staining after counting in a hemocytometer, the experiments were conducted within 2-3 h of cell dispersion.

II. 2.3. Preparation of primary cultures of gastric smooth muscle cells

The smooth muscle cells dispersed and isolated from rabbit's stomach were resuspended in DMEM culture medium containing the following antibiotics; penicillin (200 U/ml), streptomycin (200 U/ml), gentamycin (100mg/ml), amphotericin B (2.5 µg/ ml) along with 10% fetal bovine serum (DMEM-10) added through a filtered flask. The muscle cells were then plated at a concentration of 5×10^5 cells/ml and incubated at 37°C in a CO₂ water-jacketed incubator. The cells were allowed to attach to the base of the culture dish and DMEM-10 medium was replaced with fresh medium every three days for 2-3 weeks until confluence was attained. When the primary cultures of smooth muscle cell were fully confluent, they were trypsinized (0.5 mg trypsin/ml) and re-plated at a concentration of 2.5×10^5 cells/ml and further cultured under the same conditions. All the experiments were conducted with muscle cells in their first passage. Previous studies have confirmed the purity of the primary cultured cells with smooth muscle specific marker γ - actin (Teng, Murthy et al. 1998). The cultured muscle cells were starved for 24 h in serum and antibiotic free medium prior conducting any experiments.

The project entails conducting experiments on both freshly dispersed gastric smooth muscle cells and primary culture of gastric smooth muscle cells. In spite of the limitations in interpreting the experimental data obtained from cultured cells, their use was indispensable for conducting molecular studies and introducing genetic manipulations.

II. 2.1. Total RNA isolation

50-100 mg of gastric tissue was isolated from rabbit and mice stomach and transferred to a glass-Teflon homogenizer with 1 ml of TRIzol (GIBCO BRL). The tissue was properly homogenized for 1 min in the polytron. Following homogenization, the content was transferred into a 1.5 ml centrifuge tube and 0.2 ml of chloroform was added. Sample tubes were capped securely and mixed by inverting the tube for 15 sec. The tubes were incubated at room temperature for 3 min and then centrifuged at 12,000 g for approximately 15 min. At the end of centrifugation the mixture separates into two distinct layers, a lower red, phenol-chloroform phase and an upper clear aqueous phase. Since RNA appears exclusively in the upper aqueous phase, the upper phase was carefully transferred into a fresh tube. RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol followed by incubating the sample at room temperature for 2 h. The mixture was then centrifuged at 12,000 g for 10 min at 4°C and the precipitated RNA appeared to be a white pellet. The pellet was washed with 0.5 ml of 70% ethanol and centrifuged at 7500 g for 10 min at 4°C (Zhang, Li et al. 2012) and completely air/vacuum dried using an Integrated Speed-Vac System for 10 min. The dried RNA pellet was dissolved in 50-100 µl of DEPC treated water and the concentration was measured with a NanoDrop 1000.

II. 2.5. Real Time PCR

Real-time PCR was carried out on cDNA samples prepared from total RNA isolate from freshly dispersed smooth muscle cells, whole gastric tissue and also primary cultures of gastric cells. The experiment was performed using StepOne™ Real-Time PCR System (Applied Biosystem, Foster city, CA) and the intercalating dye SYBR green. Optimum PCR conditions were

determined on the gradient thermal cycler using the StepOne™ Real-Time PCR System. For each cDNA sample, 20 µl by volume of the reaction mixture was prepared with the Quantitect™ SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). The time and temperature profile used for each real-time PCR mixture is as follows: 95 °C for 5 min; 50 cycles of a series consisting of 15 s at 94 °C, 30 s at 52 °C, 30 s at 72 °C; and a final extension of 5 min at 72 °C. The optimal annealing temperature for each primer set was determined empirically. The primary sequence of the primer sets used for the reactions are tabulated as follows:

Each real-time PCR reactions were performed in triplicate. Only one PCR product was generated by each primer set and the correct molecular weight was confirmed by electrophoresis on 1.5% agarose gel containing 0.1 µg/ml ethidium bromide followed by sequencing of the individual bands to finally confirm the identity and integrity of the band. The fluorescent threshold value for each data set was calculated using the StepOne™ Real-Time PCR System software. The absence of signal in control wells containing water precluded the possibility of formation of primer-dimer.

Quantification of gene expression- Usually, two types of data quantification methods are applicable in quantitative RT-PCR (qRT-PCR). One, using an absolute quantification method that directly measures the levels of expressed genes and the other by a more relative quantification method. The absolute quantification approach uses a calibration curve that relates the PCR signal to input copy number of the target gene. On the other hand in the relative quantification method the PCR signal of the transcript of interest in a treatment group is compared to the transcript level of another sample considered as an untreated or control group.

Relative quantification of a target gene in reference to another gene also called the housekeeping gene whose expression is absolutely unfluctuating under any condition (treatment or control) is calculated on the basis of delta delta CT values (CT, also known as CP, is the cycle number at which the fluorescence generated within a reaction crosses the threshold). CT is defined as the point during the course of a reaction at which there is sufficient accumulation of amplicons. There are several mathematical models to calculate the relative expression ratio (R), based on the comparison of the diverse cycle differences. One of them includes the delta delta CT method and the efficiency corrected calculation model. In these models, the target-gene expression is normalized to the expression of one or more non-regulated housekeeping genes. The choice of an internal control to normalize the expression of the gene of interest is critical to the interpretation of experimental real-time PCR results. The housekeeping gene expression must not be influenced by the applied treatment.

Delta delta CT method

$$\Delta\Delta CT = (C_{T, \text{Tag}} - C_{T, \text{HKG}})_{\text{Treatment}} - (C_{T, \text{Tag}} - C_{T, \text{HKG}})_{\text{Control}}$$

$$R = 2^{-\Delta\Delta CT}$$

Where, HKG is the housekeeping gene and Tag, the evaluated gene.

Adhering to the criteria for choosing the reference genes in the delta delta method, GAPDH was selected as the reference gene. After normalization, the data for one gene or control group were

expressed as the fold-change in mRNA expression relative to that obtained for another gene or treatment group.

II. 2.6. Western blot analysis

Smooth muscle cells isolated from rabbit stomach or harvested from primary cultures were solubilized in Triton X-100-based lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM DTT, 100 mM NaCl, 0.5% sodium dodecyl sulphate, 0.75% deoxycholate, 1 mM PMSF, 10 µg/ml of leupeptin and 100 µg/ml of aprotinin, in the presence of protease and phosphatase inhibitors. The lysates obtained after solubilization was centrifuged at 20000 *g* for 10 min at 4 °C and the supernatant was collected and its protein concentration was determined with a Dc protein assay kit from Bio-Rad. Equal amount of protein was resolved by SDS/PAGE gel, followed by an electrophoretic transfer of the protein bands onto a nitrocellulose paper. The blots were then blocked with 5% (w/v) non-fat dried milk/TBS-T [tris-buffered saline (pH 7.6) and 0.1% Tween-20] for 1 h and then incubated overnight at 4 °C with the specific primary antibodies in TBS-T and 1% (w/v) non-fat dried milk. Following overnight incubation with primary antibodies, the blots were washed thrice with PBST and 0.1% tween and then incubated for 1 h with horseradish-peroxidase-conjugated corresponding secondary antibody (1:2000; 10 µg/ml, Pierce) in TBS-T /1% (w/v) non-fat dried milk. The immunoreactive proteins were visualized using SuperSignal Femto maximum sensitivity substrate kit (Pierce). All washing steps were performed with TBS-T. Enhanced chemiluminescence reagent identified the protein bands. Quantification of protein bands obtained on western blot was subjected to densitometric analysis by the Odyssey software using the median method for calculation of background. The average intensity obtained for each band was normalized to that of β-actin for the same lane. The band

intensity of each treatment was then calculated as a percent value normalized with the control. The percent value of the control was used for statistical analysis.

II. 2.7. Radioligand binding studies

Dispersed gastric smooth muscle cells were isolated from rabbit stomach and radio-ligand binding studies were performed using [^3H] *scopolamine* as described previously (Murthy and Makhoulf 1997). The muscle cells were suspended in HEPES medium (pH 7.4) containing 1% fetal bovine serum. For total binding, 0.5 ml of cell suspension (10^6 cells/ml) were aliquot in triplicates and incubated at room temperature for 15 min with 1 nM [^3H] *scopolamine* alone. For total binding with the specific receptor subtypes the cells were incubated with [^3H] *scopolamine* in the presence of methoctramine for total m3 receptor binding or in the presence of 4-DAMP for total m2 receptor binding. Following treatment, the bound and free radio-ligand was separated by rapid filtration under reduced pressure through 5- μm polycarbonate Nucleopore filters. The filters were extensively washed for 4 times with 3 ml of ice-cold HEPES medium containing 0.2% of bovine serum albumin. Non-specific binding was measured by treating the cells with carbachol at a concentration of 10 μM . Specific binding of the radio-ligand with the receptors was obtained by subtracting the non-specific binding from total binding.

II. 2.8. Assay for Phosphoinositide (PI) hydrolysis

The total inositol phosphates pool from muscle cells was measured by the process of anion exchange chromatography using the method of Berridge et al (Murthy and Makhoulf 1991; Murthy, Zhou et al. 2003). A volume of 10 milliliters of cell suspension were labeled with myo-2-[^3H] inositol (15 $\mu\text{Ci/ml}$) for 180 min at 31°C. Excess [^3H] inositol was removed by

centrifuging the muscle cells at 350 g for 10min. After incubating with DMSO and M β CD and treatment with CCh (0.1 μ M) and methoctramine (0.1 μ M,) the cells was placed in a shaking water bath for 1 min. Similar procedure was followed for the other two approaches, caveolin-1 siRNA targeted cultured smooth muscle cells and gastric tissue obtained from caveolin-1 KO mice. Following treatment the reaction was terminated by the addition of choloform:methanol:HCl in the ratio of 50:100:1 by volume. After adding equal volume of chloroform and water (340 μ l), the samples were vortexed and centrifuged at 1000 g for 15 min to separate the phases. The upper clear aqueous phase was applied to a column containing 1 ml of 1:1 slurry of Dowex AG-1 X8 resin (100-200 mesh in formate form) and distilled water. The column was then washed with 10 ml of distilled water followed by 10 ml solution of 5 mM sodium tetraborate and 60 mM ammonium formate to remove [3 H] glycerophosphoinositol. Total inositol phosphates were eluted by applying 6 ml of 0.8 M ammonium formate - 0.1 M formic acid. The eluates were collected in scintillation vials and counted in gel phase after addition of 10 ml of scintillation fluid. The results were expressed as counts per minute per mg of protein.

II. 2.9. Assay for adenylyl cyclase activity

Adenylyl cyclase activity was measured by the formation of the second messenger cAMP in response to the contractile agonist carbachol. Radioimmunoassay using radiolabeled [125 I] cAMP was performed to measure the level of cAMP production (Murthy and Makhlouf 1997; Murthy and Makhlouf 1998; Teng, Grider et al. 2001). Carbachol stimulates muscarinic m2 receptor acting via G α i to inhibit adenylyl cyclase activity. Carbachol induced inhibition of adenylyl cyclase activity was monitored as percent reduction of cAMP formation compared to forskolin induced cAMP levels. The experiments were performed after the cells were treated with

carbachol (CCh: 0.1 μ M) and the muscarinic m3 receptor antagonist 4-DAMP (0.1 μ M). One milliliter of cell suspension containing approximately 3×10^6 cells/ml was treated with 10 μ M of forskolin either alone or in combination with carbachol (0.1 μ M). Following treatment the reaction was terminated with cold 6% trichloroacetic acid (v/v) and vortexed vigorously. The mixture was then centrifuged and the supernatant was collected and were extracted three times with water-saturated diethyl ether to remove the trichloroacetic acid and then lyophilized and frozen at -20°C. Before radioimmunoassay, the samples were reconstituted in 50 μ l of 50mM sodium acetate (pH 6.2) and acetylated with triethylamine/acetic anhydride (2:1 v/v) for 30 min. Cyclic AMP was measured in triplicates using 100 μ l aliquots and the results was analyzed and derived from a standard curve using Prism[®] GraphPad program. The results were expressed as pmol of cAMP/mg protein.

II. 2.10. Immunokinase assay for Rho kinase and ZIP kinase activity

Rho kinase and ZIP kinase activities were measured by immunokinase assay as previously described (Murthy and Makhoulf 1998; Murthy, Zhou et al. 2001; Zhou and Murthy 2004). Muscle cells were divided into two groups, control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with M β CD (10mM) each for 30 min. After 30 min incubation cells were washed three times with smooth muscle buffer and equal amount of cells (3×10^6 cells/ml) were treated with carbachol (0.1 μ M) and Rho Kinase inhibitor, Y27632 (1 μ M) for 10 minutes and the reaction was terminated by rapid centrifugation at 25000 g for 15 mins at 4°C. The same treatment process was followed for other two approaches, caveolin-1 siRNA transfected cultured gastric muscle cells and cells obtained from caveolin-1 knockout mice. Following treatment the cells were lysed with lysis buffer containing

50mM Tris-HCl (pH 7.5), 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 10mM sodium pyrophosphate, and protease inhibitor cocktail (2 µl/ml, BD Biosciences). The homogenates were further centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant containing cytosolic protein after being transferred to a new 1.5 ml tube was mixed with 5 µl of Rho Kinase antibody and incubated for 2 hrs at 4°C.

At the completion of two hours, protein A/G agarose beads were added to each tube and the mixture was reincubated at 4°C for overnight and washed 3 times with lysis buffer. The pellet was resuspended in 50 µl of kinase buffer containing 100mM Tris-HCl (pH7.4), 1M KCl, 50mM MgCl₂, 10mM EDTA, and 1mM DTT. Twenty microlitres of Rho kinase immunoprecipitates were added to the reaction mixture containing 100 mM Tris- HCl (pH7.4), 1M KCl, 50 mM MgCl₂, 1mM DTT, 1mM ATP, and 10 µCi of [γ - ³²P] ATP (3000 Ci/mol) along with 1µg of myelin basic protein, followed by incubation for 15 mins at 37°C. Phosphorylation of myelin basic protein was absorbed onto phosphocellulose disks, and free radioactivity was removed by washing 3 times with 75mM H₃PO₄. The amount of radioactivity on the disks was measured by liquid scintillation. The results are expressed as counts per minute per milligram protein (31).

II. 2.11. Transfection of caveolin-1 siRNA into cultured gastric smooth muscle cells

Primary culture of gastric smooth cells was grown at 80-90% confluence and serum starved for overnight. The caveolin-1 specific siRNA was subcloned into the pSIREN-DNR-DsRed expression vector. The recombinant vector was diluted with Opti-MEM^R and mixed gently. After dilution of the plasmid vector into Opti-MEM the mixture was incubated for 5 min at room temperature. Similarly, the transfection agent LipofectamineTM 2000 was also diluted in opti-MEM and mixed gently. The diluted recombinant plasmid and the diluted LipofectamineTM 2000

reagent was mixed together and further incubated at room temperature for 20 min. After 20 min, the mixture were added to cultured muscle cells and kept for 48 h for successful knockdown of caveolin-1 protein. The transfection efficiency was monitored by the expression of the red fluorescent protein integrated into the vector, by TRITC filters. The control cells were transfected with vector alone. Imaging using fluorescent microscopy confirmed 80% transfection efficiency. Following each transfection method, cell lysates were collected and the protein level was confirmed by western blot analysis (Zhou, Das et al. 2003; Huang, Zhou et al. 2007).

II. 2.12. Sucrose Density Centrifugation of gastric cells

Rabbit gastric smooth muscle cells in their first passage were maintained in culture. Once the cells attained 70-80 % confluence, the medium was aspirated and the plates were washed with ice cold PBS. At the end of third wash, PBS was completely removed and 1 ml of 1% Triton-X 100 buffer was applied to each plate enough to cover the plates. The cells were then scrapped off using a cell scraper and transferred into a prechilled Dounce (glass-glass) homogenizer. Following homogenization, the cell lysate was subjected to sucrose density centrifugation to separate caveolar and non- caveolar fraction. The principle of separation was based on the unique lipid enrichment of the caveolar fraction such as sphingolipids and cholesterol, which makes these fractions more buoyant compared to other cellular content.

After homogenization, 2 ml of the sample was mixed with 2 ml of 90 % MBS/Triton X-100 buffer in an ultracentrifuge tube to prepare a sucrose layer of 45%. 4 ml each of 35% and 5 % sucrose prepared in MBS/Triton X-100 buffer was carefully layered on top of the 45% sucrose layer. Following the preparation of the sucrose density layer, the tube was perfectly balanced and centrifuged for 16-20 h at 39,000 rpm at 4⁰C in a SW41Ti rotor (Beckman). After the

centrifugation, the tubes were carefully removed from the bucket and the samples were collected from top to bottom in 1 ml fractions. Each fraction collected was analyzed by SDS-PAGE. The caveolar fractions were probed with a caveolin-1 specific antibody (Murthy and Makhoulf 2000; Ostrom and Insel 2006).

II. 2.13. Receptor Internalization by radioligand binding studies

Dispersed gastric smooth muscle cells and cultured cells were used to study the effect of caveolin-1 on muscarinic m2 and m3 receptor internalization. Receptor internalization was measured by radio-ligand binding studies using [3H] *scopolamine* (Murthy, Grider et al. 2000). The muscle cells were suspended in HEPES medium (pH 7.4) containing 1% fetal bovine serum. For total binding, 0.5 ml of cell suspension (10^6 cells/ml) were aliquot in triplicates and incubated at room temperature for 15 min with 1 nM [3H] *scopolamine* alone or with [3H] *scopolamine* and 0.1 μ M carbachol in the presence of muscarinic m2 and m3 receptor antagonists, methoctramine and 4-DAMP respectively. Receptor internalization was induced by pre-treating the cells with CCh (0.1 μ M) for 20 min. Following incubation the bound and free radio-ligand was separated by rapid filtration under reduced pressure through 5 μ m polycarbonate Nucleopore filters. The filters were extensively washed for 4 times with 3 ml of ice-cold HEPES medium containing 0.2% of bovine serum albumin. Total binding was again measured using similar protocol after receptor internalization. Non-specific binding was calculated by treating the cells with CCh at a concentration of 10 μ M (100 times). Specific binding of the radio-ligand for the receptors was obtained by subtracting the non-specific binding from total binding.

II. 2.14. Measurement of smooth muscle cell contraction by scanning micrometry

Freshly dispersed gastric smooth muscle cells were incubated with DMSO and methyl β -cyclodextrin (M β CD) (10 mM) separately each for 30 min. Dispersed cells were also isolated from caveolin-1 KO and WT mice. Following incubation and cell isolation, aliquots of 0.4 ml of cell suspension were treated with CCh (0.1 μ M) in the presence of m2 receptor antagonist methoctramine (0.1 μ M). After treatment the reactions were terminated using 8% acrolein at a final concentration 0.1% that helps to kill and fix the cells without affecting the cell length. The resting cell length was determined in control experiments with muscle cell suspension treated with 100 μ l of 0.1% bovine serum albumin without the agonists and antagonist. The peak contraction was derived from the concentration response curve after various treatments. The mean length of 50 smooth muscle cells treated with CCh and methoctramine were measured by scanning micrometry as described previously (Murthy, Zhou et al. 2003) and compared to the mean length of untreated cells. The contractile response was expressed as the percent decrease in mean cell length compared to the control cell length.

II. 2.15. In-cell western

Culture of smooth muscle cells were grown in 96 well plate (0.1 X 10⁶ cells/well) and treated with carbachol (CCh: 0.1 μ M) and methoctramine (0.1 μ M). Following treatment the medium was aspirated and washed three times with 1XPBS and immediately fixed by adding 150 μ l of fixing solution to each well. After 20 min of fixation at room temperature without shaking, the solution was removed and 200 μ l of Triton washing solution was added to each well and placed on a shaker for 5 min. The washing step was repeated for 4 more times. Following extensive washing 150 μ l of Odyssey blocking buffer was added to each well and incubated for 2 h at

room temperature on a shaker. After blocking, the primary antibody (1:200 dilution) was dissolved in 50 µl of blocking buffer and added to each well. The plate was incubated with the primary antibody on a shaker at a gentle speed at 40C for 2 h. Following incubation with primary antibody the wells were washed with Tween washing solution for five times and 50 50 µl of the specific secondary antibody containing Sapphire and DRAQ5 for background detection was added for 1 h at room temperature. The plate was washed 5 times with 200 µl of Tween washing solution and scanned under Licor-Odyssey imaging system using both 700 and 800 nm channels.

Statistical Analysis

The results were expressed as means + S.E of n experiments and analysed for statistical significance using Student t -test for paired and unpaired values. Each experiment was performed on cells and tissues obtained from different animals. Difference of responses among different groups were tested and confirmed for significance using Fisher's protected least significance difference test. The results were analysed using GraphPad software, San Diego, CA. A probability of $P < 0.05$ was considered significance.

CHAPTER III: RESULTS

III. 1. Signaling transduction by muscarinic receptors

Molecular, pharmacological and immunohistochemical studies demonstrated that at least five different muscarinic acetylcholine (ACh) receptors (m1-m5) are expressed in mammalian tissues and their expression is tissue- and species-specific. In vascular and visceral smooth muscle ACh interacts mainly with m2 and m3 receptors; m3 receptors are coupled via G_q with phosphoinositide (PI) hydrolysis and inositol 1,4,5-trisphosphate (IP_3)-dependent Ca^{2+} release, whereas m2 receptors are coupled via G_i with inhibition of adenylyl cyclase (Migeon, Thomas et al. 1995; Schramm, Arjona et al. 1995; Morel, Macrez et al. 1997; Murthy, Zhou et al. 2003; Tran, Matsui et al. 2006). Our initial studies characterized the expression of muscarinic receptors and the receptor-specific signaling pathways activated by carbachol (acetylcholine mimetic) in rabbit gastric smooth muscle cells.

III. 1a. Expression of muscarinic m2 and m3 receptors in gastric smooth muscle

Specific primers for muscarinic m1, m2, m3 and m4 receptors were designed based on the conserved sequences in human, rabbit, rat, and mouse cDNAs and used for identification of muscarinic receptor mRNA expression. Muscarinic m2 and m3, but not m1 and m4 receptors were detected by RT-PCR on RNA extracted from cultures of gastric smooth muscle cells in first passage (Fig. 1). When experiments were done in the absence of reverse transcriptase (RT), there was no amplification of either m2 or m3 receptors. Use of m1, m2, m3 and m4 receptor cDNA's as positive control resulted in an amplification of mRNA with receptor specific primers. As shown previously, the use of confluent cultures of smooth muscle in first passage ensured the absence of neural, endothelial, or interstitial cell contaminants (Teng, Murthy et al. 1998) and the

presence of PCR product in cultured muscle cells demonstrate the specific expression of m2 and m3 mRNA in smooth muscle cells.

Further confirmation for the expression of m2 and m3 receptors was obtained by western blot analysis using selective antibody to m2 and m3 receptors. The results demonstrated the expression of both m2 and m3 receptors of predicted size 52 kDa and 66 kDa respectively in the homogenates of isolated smooth muscle cells (Fig. 1). The results are consistent with the selective expression of m2 and m3 receptors in vascular and other visceral smooth muscle (Lin, Kajimura et al. 1997; Belmonte 2005; Gericke, Sniatecki et al. 2011).

Selective expression of m2 and m3 receptors in gastric smooth muscle cells was further confirmed by radioligand binding studies using [3 H]Scopolamine as muscarinic agonist. Specific binding was calculated as the difference between the total binding in the absence of acetylcholine (ACh) and non-specific binding in the presence of (10 μ M) ACh. Total binding was $2,105 \pm 302$ cpm/mg protein and the non-specific binding was $22 \pm 4\%$ of the total binding (456 ± 65 cpm/mg protein). Incubation of cells with the selective m2 receptor antagonist, methoctramine (0.1 μ M) inhibited specific binding by $69 \pm 3\%$, whereas incubation of cells with the selective m3 receptor antagonist 4-DAMP (0.1 μ M) inhibited specific binding by $32 \pm 4\%$. Incubation of cells with both methoctramine and 4-DAMP abolished the specific binding of [3 H]Scopolamine (Fig. 2). The results suggest that the gastric smooth muscle cells express only m2 and m3 receptors and the expression of m2 receptors are more abundant than m3 receptors. The radioligand binding studies are consistent with the selective expression of m2 and m3 receptor mRNA and proteins in these cells.

III. 2. Signaling pathways coupled to m2 and m3 receptors in gastric smooth muscle

Previous radioligand binding and pharmacological studies in gastrointestinal smooth muscle cells had demonstrated a high selectivity of methoctramine for m2 receptors and 4-DAMP for m3 receptors (Murthy and Makhlouf 2000). This made it possible to use these antagonists to examine signaling pathways initiated by m2 and m3 receptors. The signaling pathways activated by m2 receptors are examined in response to carbachol (CCh: 0.1 μ M) in the presence of 4-DAMP (0.1 μ M), whereas the signaling pathways activated by m3 receptors are examined in response to CCh in the presence of methoctramine (0.1 μ M). The concentrations of CCh and antagonists were selected based on our previous studies in isolated gastrointestinal smooth muscle cells. Since previous studies have demonstrated that m3 receptors are coupled with G_q / G_{13} and m2 receptors are coupled to pertussis toxin (PTx)-sensitive G_i (Murthy 2008), in the present study, PTx was also used as an additional tool to identify pathways initiated by m2 and m3 receptors.

III. 2a. Gq-coupled muscarinic m3 receptor signaling

Acetylcholine was shown previously to stimulate PI hydrolysis, IP_3 formation and Ca^{2+} release via G_q in dispersed intestinal smooth muscle cells (Murthy 2008). In the present study, CCh-stimulated PI-specific phospholipase C (PLC) activity (PI hydrolysis) in the presence of m2 receptor antagonist methoctramine (0.1 μ M) was measured as readout of m3-coupled G_q activity. Incubation of freshly dispersed smooth muscle cells from rabbit stomach with CCh (0.1 μ M) in the presence of methoctramine (0.1 μ M) caused a significant increase in PI hydrolysis ($3,075 \pm 532$ cpm/mg protein) compared to the basal PI hydrolysis (400 ± 83 cpm/mg protein). The increase in PI hydrolysis was significantly inhibited in the presence of selective m3 receptor

antagonist 4-DAMP (843 ± 121 cpm/mg protein; $84 \pm 5\%$ decrease compared to control CCh response, $p < 0.001$). Pretreatment of cells with U73122 ($1 \mu\text{M}$), a selective inhibitor of PI hydrolysis blocked CCh-induced increase in PI hydrolysis ($87 \pm 6\%$ inhibition, $p < 0.001$) (Fig. 3).

III. 2b. G_{13} -coupled muscarinic m3 receptor signaling

Acetylcholine was shown previously to stimulate RhoA and RhoA-dependent Rho kinase activity via $G\alpha_{13}$ in dispersed intestinal smooth-muscle cells (Murthy, Zhou et al. 2003). In the present study, stimulation of Rho kinase and zipper interacting protein (ZIP) kinase activity in response to CCh was measured as readout of m3-coupled G_{13} activity.

Rho kinase. Incubation of freshly dispersed gastric smooth muscle cells with CCh ($0.1 \mu\text{M}$) for 10 min caused a significant stimulation of Rho kinase activity ($33,582 \pm 2412$ cpm/mg protein) compared to basal activity ($9,006 \pm 1683$ cpm/mg protein). Pretreatment of cells with Y27632 ($1 \mu\text{M}$), the specific inhibitor of Rho kinase blocked the increase in Rho kinase activity ($88 \pm 5\%$ inhibition, $p < 0.001$). Pretreatment of cells with 4-DAMP ($0.1 \mu\text{M}$) also blocked the increase in Rho kinase activity in response to CCh ($12,649 \pm 1443$ cpm/mg protein; $85 \pm 7\%$ decrease compared to CCh response, $p < 0.001$), whereas pretreatment of cells with methoctramine ($0.1 \mu\text{M}$) had no significant effect on the increase in Rho kinase activity ($30,829 \pm 3406$ cpm/mg protein; $9 \pm 6\%$ inhibition) (Fig. 4). These results suggest that CCh-stimulated Rho kinase activity was mediated via m3 receptors.

ZIP kinase. Incubation of freshly dispersed smooth muscle cells with CCh ($0.1 \mu\text{M}$) for 10 min caused a significant stimulation of ZIP kinase activity ($21,328 \pm 2757$ cpm/mg protein) compared to basal activity ($4,446 \pm 1207$ cpm/mg protein). Pretreatment of cells with Y27632 ($1 \mu\text{M}$), the specific inhibitor of Rho kinase significantly inhibited the increase in ZIP kinase

activity ($7,467 \pm 1397$ cpm/mg protein; $83 \pm 6\%$ inhibition, $p < 0.001$). These results suggest that ZIP kinase activity is downstream of Rho kinase activity. Pretreatment of cells with 4-DAMP blocked the increase in ZIP kinase activity in response to CCh ($7,610 \pm 1192$ cpm/mg protein; $80 \pm 5\%$ decrease compared to CCh response, $p < 0.001$), whereas pretreatment of cells with methoctramine ($0.1 \mu\text{M}$) had no significant effect on the increase in ZIP kinase activity ($21,549 \pm 2868$ cpm/mg protein) (Fig. 5). These results suggest that CCh-stimulated ZIP kinase activity was mediated via m_3 receptors and is dependent on stimulation of Rho kinase activity.

III. 2c. G_i -coupled muscarinic m_2 receptor signaling

Previous studies in vascular and visceral smooth muscle have shown that ACh interacts with G_i -coupled m_2 receptors and inhibits adenylyl cyclase activity (Zhang, Horowitz et al. 1991; Murthy and Makhoulf 1997; Hakonarson and Grunstein 1998). In the present study, inhibition of forskolin-stimulated cAMP formation was measured as readout of m_2 -coupled G_i activity. Forskolin ($10 \mu\text{M}$) caused an increase in cAMP formation in dispersed muscle cells (24.0 ± 2.3 pmol/mg protein) compared to basal levels of cAMP (2.5 ± 0.5 pmol/mg protein). The increase in cAMP by forskolin ($10 \mu\text{M}$) was significantly inhibited (8.0 ± 1.7 pmol/mg protein; $74 \pm 3\%$ inhibition, $p < 0.001$) in the presence of CCh ($0.1 \mu\text{M}$), and the inhibition was significantly reversed by pretreatment of cells with methoctramine ($0.1 \mu\text{M}$) ($9 \pm 4\%$ inhibition), whereas pretreatment of cells with 4-DAMP had no significant effect on the inhibition of cAMP formation ($67 \pm 7\%$ inhibition) (Fig. 6). Pretreatment of muscle cells with PTx (400 ng/ml) abolished the inhibitory effect of CCh on cAMP formation consistent with the stimulation of G_i proteins by m_2 receptor activation (23.5 ± 3.2 pmol/mg protein; $4 \pm 2\%$ inhibition, NS) (Fig. 6).

III. 2d. m3 receptor-mediated muscle contraction

As shown previously in intestinal muscle, treatment of dispersed muscle cells with CCh (0.1 μ M) for 30 s caused muscle contraction ($28 \pm 3\%$ decrease in muscle cell length from basal muscle cell length of $112 \pm 4 \mu$ m) (Murthy, Zhou et al. 2003). Pretreatment of muscle cells with 4-DAMP (0.1 μ M) abolished CCh-induced contraction ($4 \pm 2\%$ decrease in cell length), whereas pretreatment of cells with methoctramine (0.1 μ M) had no significant effect on muscle contraction ($25 \pm 3\%$ decrease in cell length) (Fig. 7). These results are consistent with the previous reports demonstrating selective involvement of m3 receptors in smooth muscle contraction. More than 90% of the contraction in response to CCh was blocked in gastrointestinal and urinary bladder muscle from m3 receptor knockout (KO) mice, whereas contraction was minimally affected in m2 receptor KO mice (Stengel, Yamada et al. 2002).

In summary, gastric muscle cells express both m2 and m3 receptors: expression of m2 receptors was more abundant than m3 receptors. Carbachol, acting via m3 receptors caused stimulation of PI hydrolysis, Rho kinase and ZIP kinase activity, and induced muscle contraction, and acting via m2 receptors caused inhibition of forskolin-stimulated cAMP formation (Fig. 8.).

Fig. 1. Selective expression of muscarinic m2 and m3 receptors in gastric smooth muscle cells.

Upper panel: mRNA Expression: Total RNA was isolated from cultured gastric smooth muscle cells (first passage) and reverse transcribed using 2µg of total RNA. The cDNA was amplified with specific primers for different muscarinic receptor subtypes m1 to m4. Experiments were done in the presence or absence of reverse transcriptase (RT). PCR products corresponding to m2 and m3 receptor were obtained with the specific primers and further confirmed with control cDNAs. Primers corresponding to m1 and m4 receptors did not amplify any specific bands from RNA isolated from muscle cells, but amplified a specific product from control cDNA. Results show representative PCR products separated by electrophoresis in agarose gel containing ethidium bromide and visualized under a chemiImager fluorescence system.

Lower panel: Protein expression: Freshly dispersed muscle cells were homogenized in lysis buffer and lysates containing equal amounts of total proteins were separated on SDS-PAGE and the expression of muscarinic receptors was analysed using selective antibody to m2 (1:1000) and m3 (1:1000) receptors and the band corresponding to 52 kDa and 66 kDa, respectively was detected by chemiluminiscence. The representative western blot shows the presence of muscarinic m2 and m3 receptors.

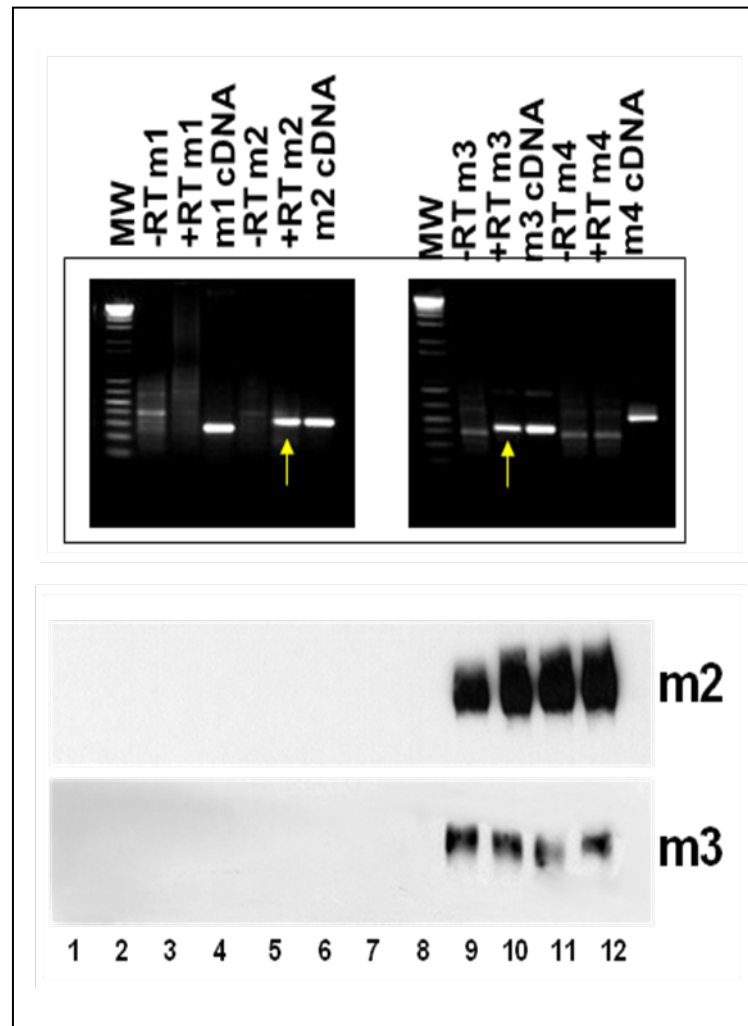


Fig.2. Selective expression of muscarinic m2 and m3 receptors by radioligand binding studies.

Freshly dispersed gastric smooth muscle cells were incubated with [^3H]scopolamine for 15 minutes at room temperature and the amount of radioactive [^3H]scopolamine bound to cells was measured by liquid scintillation. Specific binding was measured as the difference between the total binding (cpm/mg protein) measured in the absence of 10 μM carbachol (CCh) and non-specific binding (cpm/mg protein) measured in the presence of 10 μM CCh. Cells were incubated in the presence of 4-DAMP (0.1 μM) to measure binding to m2 receptors or in the presence of methoctramine (0.1 μM) to measure binding to m3 receptors. Results are expressed as % specific binding. Values are means \pm S.E of 5 experiments.

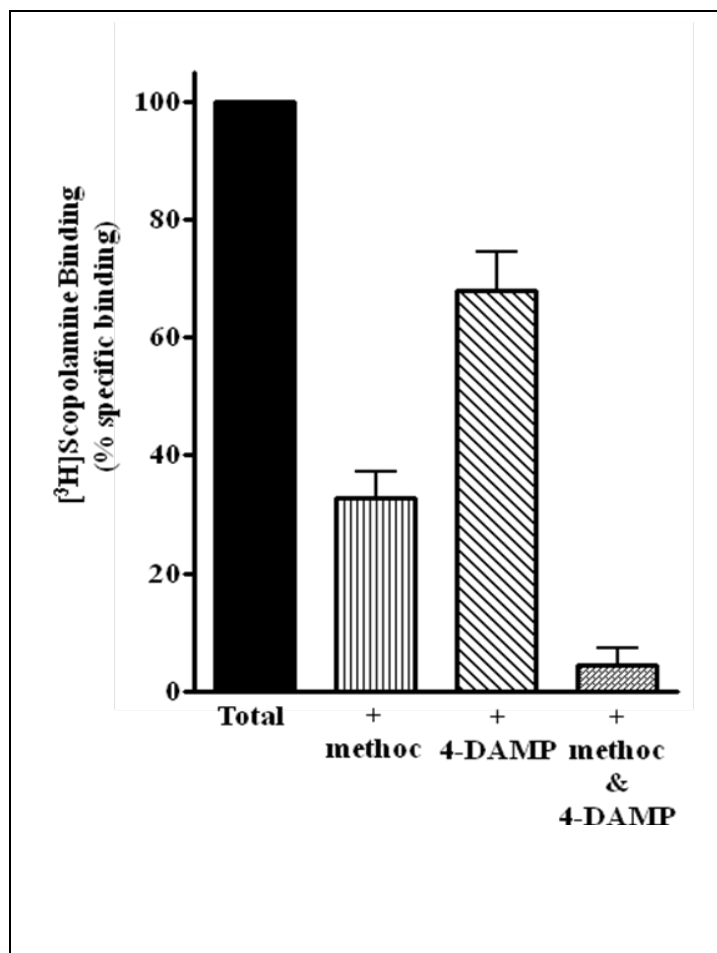


Fig. 3. m3 receptor-dependent stimulation of phosphoinositide (PI) hydrolysis in gastric muscle

Freshly dispersed muscle cells labelled with myo-[^3H]inositol were incubated with carbachol (CCh; 0.1 μM) plus methoctramine (0.1 μM) for 60 seconds. In some experiments cells were pretreated with 4-DAMP (0.1 μM) or the selective PI hydrolysis inhibitor U73122 (1 μM) for 10 min. Total [^3H]inositol phosphates were separated by ion-exchange chromatography and radioactivity counted by liquid scintillation. Results are expressed as total [^3H]inositol phosphate formation in cpm/mg protein. Values are means \pm S.E. of 4 experiments. ** Significant increase in response to CCh ($P < 0.001$).

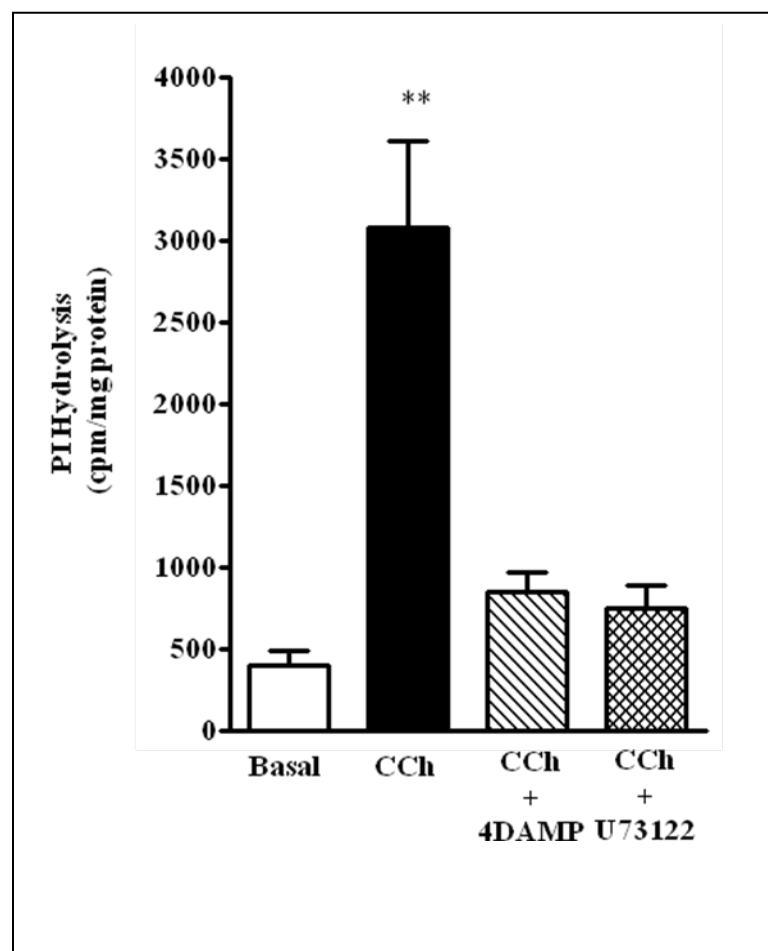


Fig. 4. m3 receptor-dependent stimulation of Rho Kinase activity in gastric muscle.

Freshly dispersed gastric muscle cells were incubated with carbachol (CCh, 0.1 μ M) for 10 min in the presence or absence of 4-DAMP (0.1 μ M), methoctramine (0.1 μ M), or the selective Rho kinase inhibitor Y27632 (1 μ M). Rho kinase activity was measured using [γ - 32 P]ATP by immunokinase assay. Rho kinase activity was stimulated by CCh and inhibited by 4-DAMP and Y27632, but not by methoctramine. Results are expressed as cpm/mg protein. Values are means \pm S.E of 5 experiments. ** Significant increase in response to CCh ($P < 0.001$).

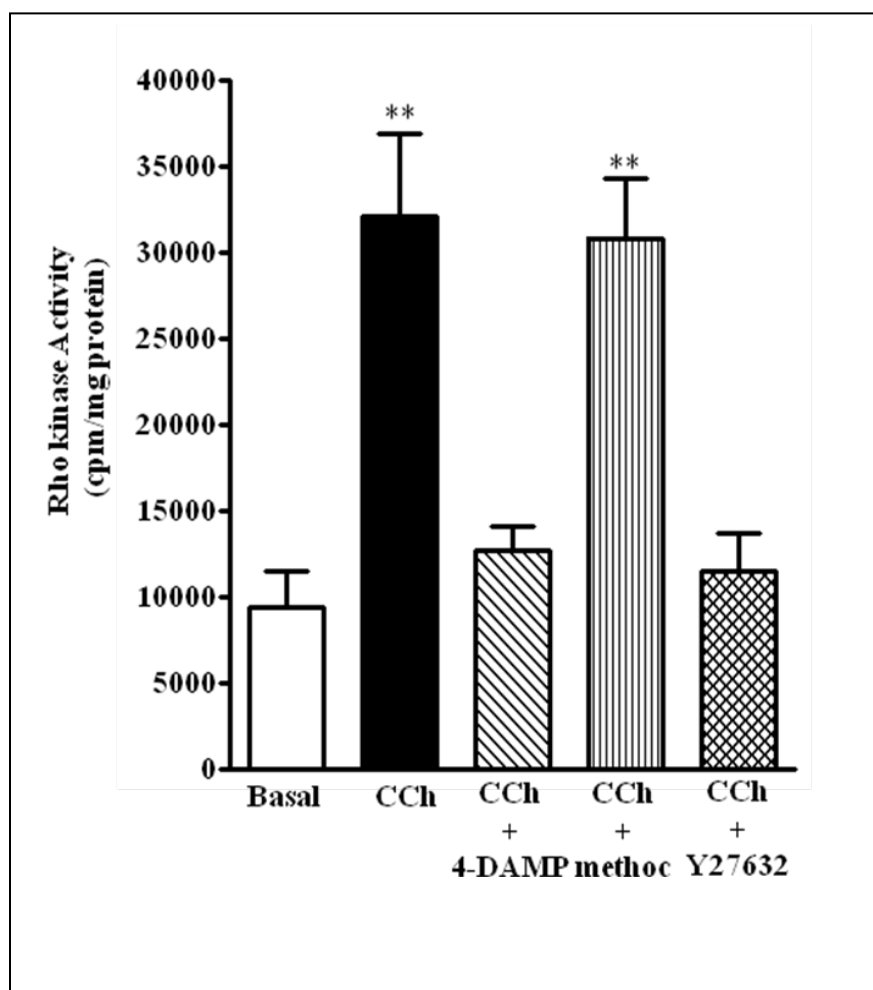


Fig. 5. m3 receptor-dependent stimulation of zipper interacting protein (ZIP) Kinase activity in gastric muscle.

Freshly dispersed gastric muscle cells were incubated with carbachol (CCh, 0.1 μ M) for 10 min in the presence or absence of 4-DAMP (0.1 μ M), methoctramine (0.1 μ M), or the selective Rho kinase inhibitor Y27632 (1 μ M). ZIP kinase activity was measured using [γ -³²P]ATP by immunokinase assay. ZIP kinase activity was stimulated by CCh and inhibited by 4-DAMP and Y27632, but not by methoctramine. Results are expressed as cpm/mg protein. Values are means \pm S.E of 5 experiments. ** Significant increase in response to CCh (P<0.001).

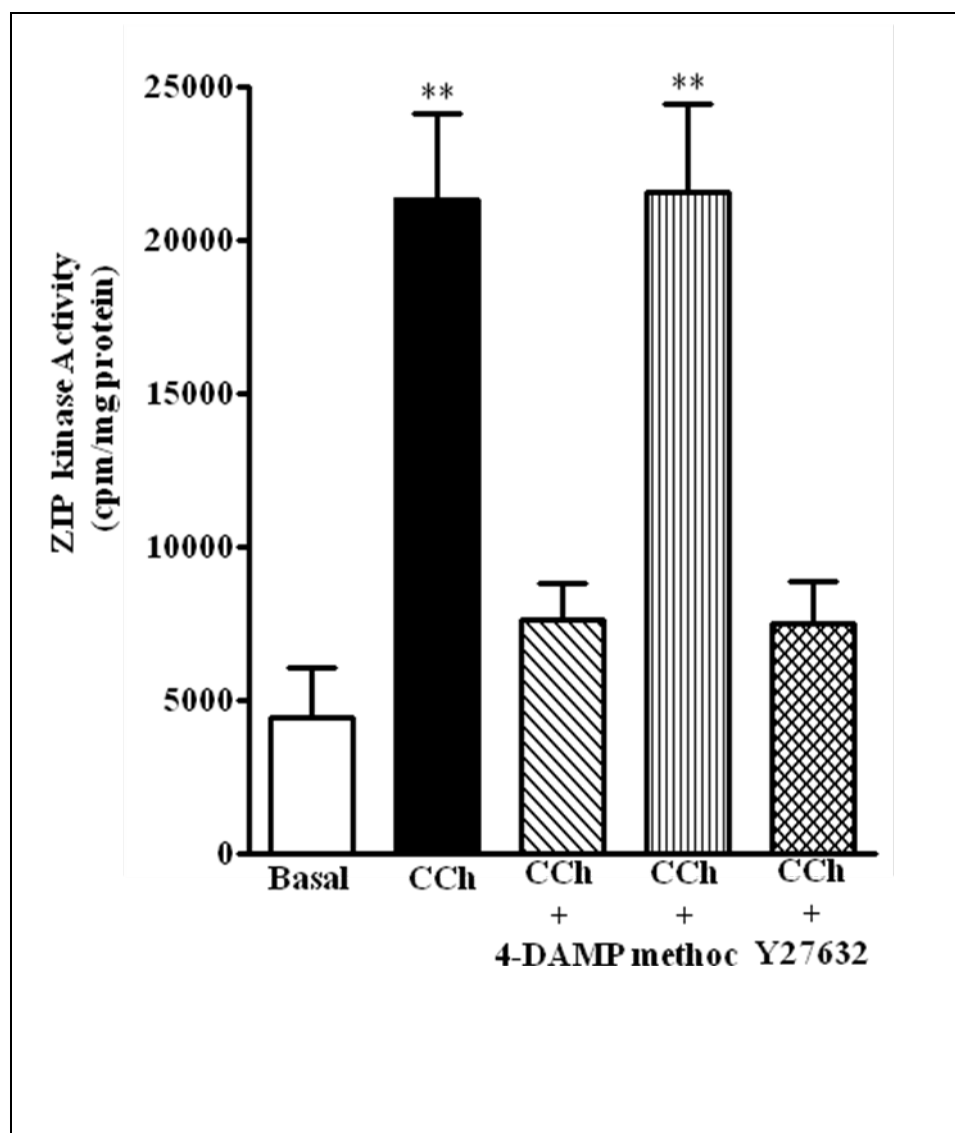


Fig. 6. m2 receptor-dependent inhibition of adenylyl cyclase activity in gastric muscle.

Adenylyl cyclase activity was measured as cAMP formation in freshly dispersed muscle cells in the presence of 100 μ M isobutylmethylxanthine (IBMX). Muscle cells were treated with forskolin (FSK, 10 μ M) in the presence or absence of CCh (0.1 μ M) for 60 s and cAMP was measured by radioimmunoassay. In some experiments, cells were pre treated with 4-DAMP or methoctramine for 10 min or pertussis toxin (400 ng/ml) for 60 min, and then treated with forskolin (10 μ M) and CCh for 60 s. Results are computed from a standard curve using Prizm[®] and expressed as pmol cAMP/mg protein. Values are means \pm S.E. of 4 experiments. ** Significant inhibition of forskolin-stimulated cAMP formation ($P < 0.001$).

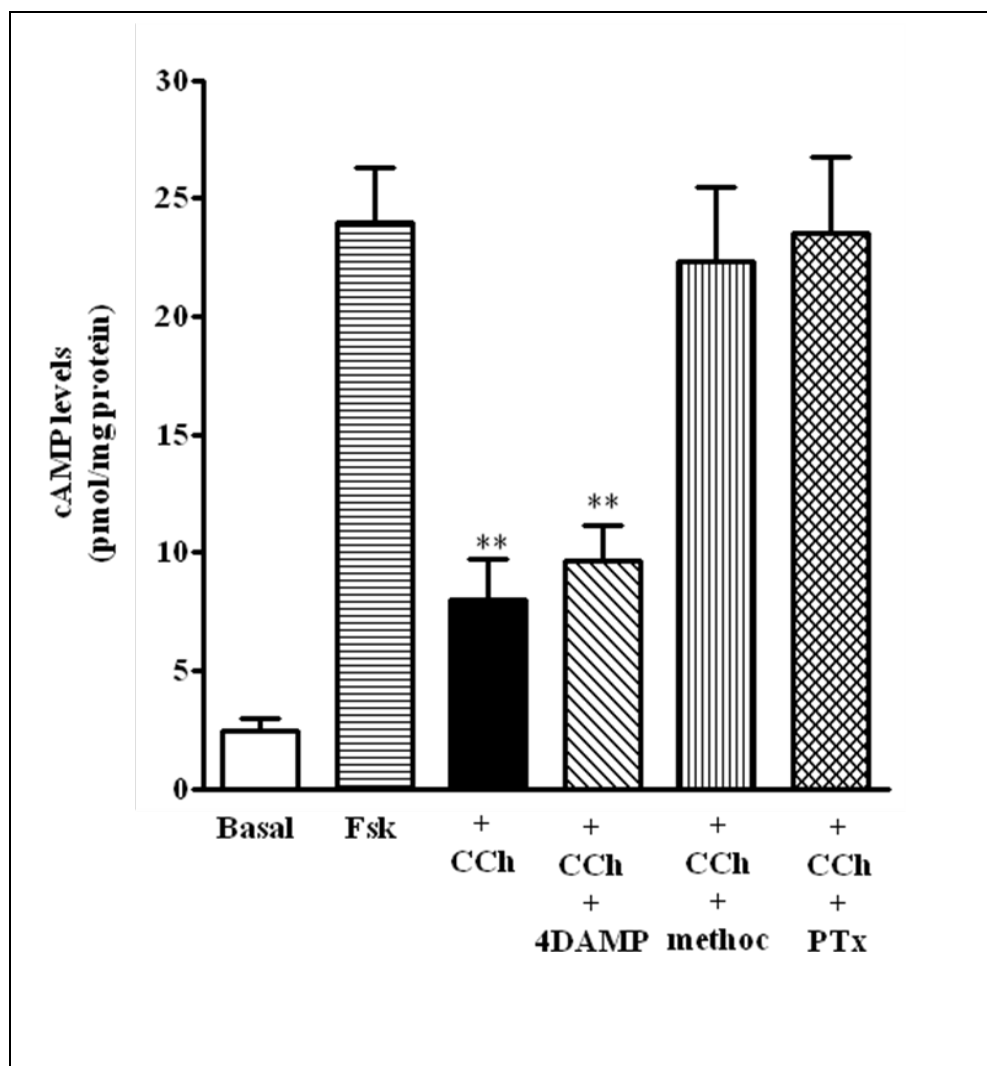


Fig.7. m3 receptor-dependent muscle contraction in gastric muscle.

Freshly dispersed gastric smooth cells were treated separately with CCh (0.1 μ M) for 30 s. The mean length of 50 muscle cells was measured by scanning micrometry and was compared with the length of untreated muscle cells. In some experiments, cells were pre treated with 4-DAMP or methoctramine for 10 min or pertussis toxin (PTx, 400 ng/ml) for 60 min, and then treated with CCh for 30 s. Results were expressed as percent decrease in cell length from control length (112 ± 4 μ m). Values are means \pm S.E of 6 experiments. ** Significant decrease in muscle cell length in response to CCh ($P < 0.001$).

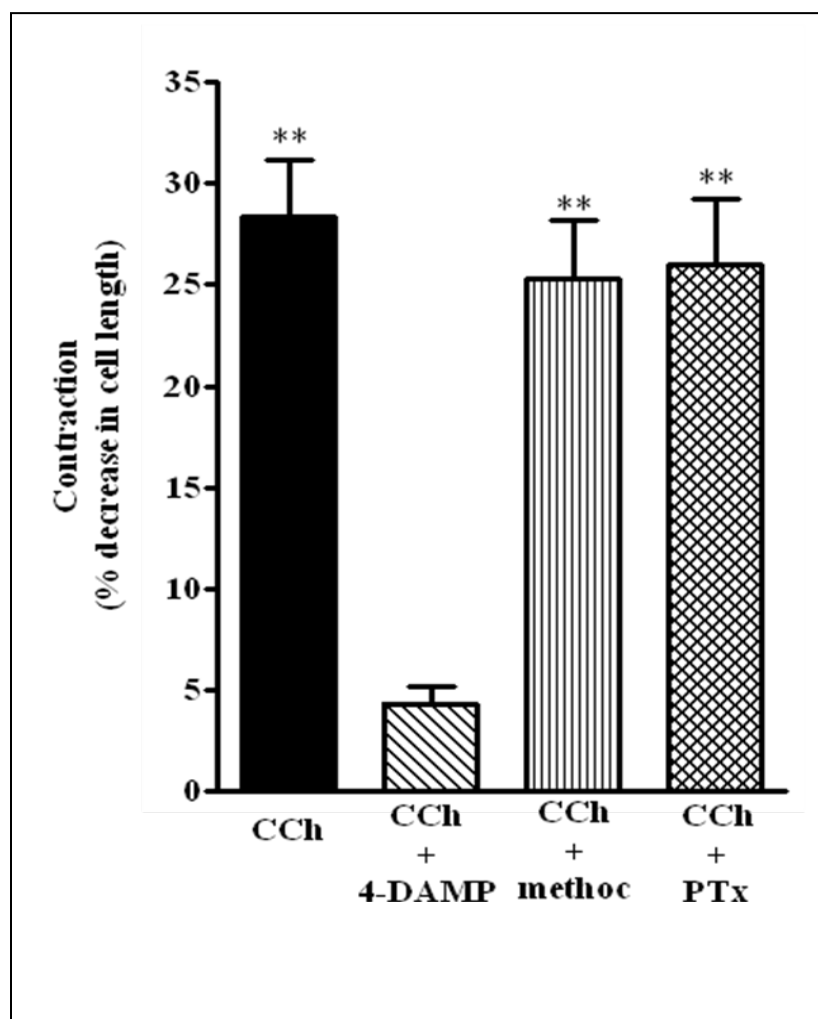
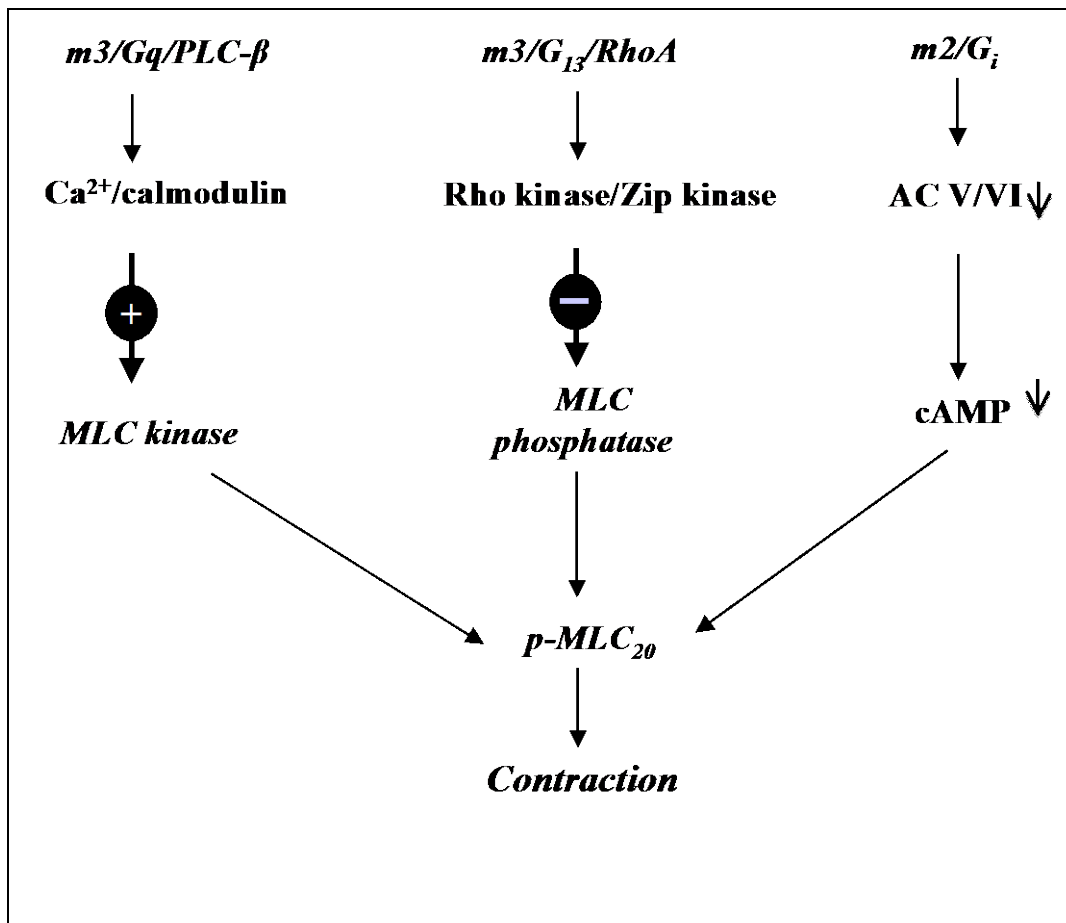


Fig 8: Signaling pathways activated by m2 and m3 receptors in gastric muscle cells.

Gastric muscle cells express m2 and m3 receptors. m2 receptors are coupled to inhibition of adenylyl cyclase (AC) activity and cAMP formation via a pertussis toxin (PTx)-sensitive G protein, whereas m3 receptors are coupled to stimulation of PI hydrolysis and Rho kinase and ZIP kinase activity via PTx-insensitive G protein(s), and muscle contraction.



III. 3. Regulation of G_q-coupled muscarinic m3 receptor signaling by caveolae and caveolin-1.

Mature contractile smooth muscle is characterized by the presence of abundant caveolae. Recent *in vitro* and *in vivo* studies from several investigators suggest that caveolae and caveolins modulate vascular and visceral smooth muscle function (Hardin and Vallejo 2006). The role of caveolae/caveolin-1 in the regulation of muscarinic signaling was examined using three complimentary approaches: a) methyl β -cyclodextrin (M β CD) treatment to deplete cholesterol in dispersed muscle cells, b) caveolin-1 siRNA to suppress caveolin-1 expression in cultured muscle cells, and c) caveolin-1 knockout (KO) mice.

Expression of caveolin-1 and caveolin-3 were examined by qRT-PCR using specific primers. mRNA expression of caveolin-1 was nearly 13-fold higher than caveolin-3 in gastric smooth muscle (Fig. 9). This is consistent with previous studies in both visceral and airway smooth muscle showing that the relative mRNA expression levels were 15:1:1 for caveolin-1, caveolin-2 and caveolin-3, respectively (Shakirova, Bonnevier et al. 2006).

III. 3a. Inhibition of carbachol-stimulated PI hydrolysis by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells labeled with [³H]myo-inositol were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. Previous studies have demonstrated that treatment of several types of cells with M β CD depletes cholesterol and disrupts caveolae (Patel, Murray et al. 2008). After 30 min, cells were treated with CCh (0.1 μ M) for 1 min in the presence of the m2 receptor antagonist methoctramine (0.1 μ M) and PI hydrolysis was measured as described in Methods. The basal

levels of PI hydrolysis in both groups are similar (366 ± 88 cpm/mg protein in control cell vs. 305 ± 62 cpm/mg protein in cells treated with M β CD). Treatment of control cells with CCh (0.1 μ M) caused a significant increase in PI hydrolysis ($2,566 \pm 394$ cpm/mg protein; $603 \pm 54\%$ increase above basal). Carbachol (0.1 μ M) also caused an increase in PI hydrolysis in cells treated with M β CD ($1,381 \pm 95$ cpm/mg protein; $352 \pm 38\%$ increase above basal); however, the increase was significantly attenuated ($42 \pm 3\%$ inhibition, $p < 0.01$) compared to CCh response in control cells (Fig. 10). The selective PI-specific PLC inhibitor, U73122 (1 μ M) blocked CCh-stimulated PI hydrolysis in both control cells and in cells treated with M β CD to a similar extent (Fig. 10). These results suggest that caveolae positively regulates G_q-mediated PI hydrolysis in gastric smooth muscle.

III. 3b. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Suppression of caveolin-1 expression was verified by western blot analysis and immunofluorescence. After plasmid transfection, cultured cells were labeled with [³H] myo-inositol, and then treated with the contractile agonist CCh (0.1 μ M) in the presence of m2 receptor antagonist methoctramine (0.1 μ M) for 1 min to measure PI hydrolysis. The basal levels of PI hydrolysis in both groups were similar (396 ± 75 cpm/mg protein in control cell vs. 343 ± 113 cpm/mg in cells treated with caveolin-1 siRNA). Treatment of control cells with CCh (0.1 μ M) caused a significant increase in PI hydrolysis ($2,813 \pm 288$ cpm/mg protein; $610 \pm 8\%$ increase above basal, $p < 0.001$). Carbachol also caused an increase in PI hydrolysis in cells transfected with caveolin-1 siRNA ($1,470 \pm 243$ cpm/mg protein; $328 \pm 5\%$ increase above basal); however,

the increase was significantly attenuated ($46\pm 4\%$ inhibition, $p<0.01$) compared to CCh response in control cells (Fig. 11). The selective PI-specific PLC inhibitor, U73122 ($1\text{ }\mu\text{M}$) blocked CCh-stimulated PI hydrolysis in both control cells and in cells transfected with caveolin-1 siRNA (Fig. 11). These results suggest that caveolin-1 positively regulates G_q -mediated PI hydrolysis in gastric smooth muscle.

III. 3c. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 KO.

Gastric muscle was isolated from wild type and caveolin-1 KO mice and labeled with [^3H] myo-inositol for 4 h and then treated with CCh ($0.1\text{ }\mu\text{M}$) in the presence of m2 receptor antagonist methoctramine ($0.1\text{ }\mu\text{M}$) for 1 min to measure PI hydrolysis. The basal levels of PI hydrolysis in gastric muscle from wild type (WT) and caveolin-1 KO mice were similar (457 ± 104 cpm/mg protein in WT mice vs. 384 ± 97 cpm/mg protein in caveolin-1 KO mice). Treatment of muscle from wild type mice with CCh caused a significant increase in PI hydrolysis ($2,714\pm 191$ cpm/mg protein: $500\pm 15\%$ increase above basal, $p<0.001$). Carbachol also caused an increase in PI hydrolysis in muscle from caveolin-1 KO mice ($1,577\pm 218$ cpm/mg protein; $310\pm 18\%$ increase above basal, $p<0.001$), however, the increase was significantly attenuated ($38\pm 7\%$ inhibition, $p<0.01$) compared to CCh response in wild type mice (Fig. 12). U73122 ($1\text{ }\mu\text{M}$) blocked CCh-stimulated PI hydrolysis in both wild type and caveolin-1 KO mice. These results provide conclusive evidence that caveolin-1 positively regulates G_q -mediated PI hydrolysis in gastric muscle.

III. 3d. Inhibition of carbachol-stimulated initial MLC₂₀ phosphorylation and muscle contraction by M β CD and caveolin-1 KO.

Previous studies in gastrointestinal muscle have shown that initial contraction is mediated by stimulation of PI hydrolysis, generation of IP₃, IP₃-dependent Ca²⁺ release, and Ca²⁺/calmodulin-dependent activation of MLCK and phosphorylation of MLC₂₀ at Ser¹⁹ (Murthy 2006). Since there is decrease in PI hydrolysis by disruption of caveolae with M β CD, we tested the effect of M β CD on CCh-induced MLC₂₀ phosphorylation and contraction in freshly dispersed muscle cells. MLC₂₀ phosphorylation was measured by in-cell western using phospho-specific (Ser¹⁹) antibody. Treatment of cells with CCh (0.1 μ M) significantly increased MLC₂₀ phosphorylation (210 \pm 36% increase, $p < 0.001$). Carbachol also caused an increase in MLC₂₀ phosphorylation in cells treated with M β CD (114 \pm 35% increase, $p < 0.001$); however, the increase was significantly attenuated (46 \pm 9% inhibition, $p < 0.01$) compared to CCh response in control cells (Fig. 13).

Treatment of cells with M β CD (10 mM) had no significant effect on basal muscle cell length (control: 115 \pm 4 μ m vs. M β CD treated: 111 \pm 5 μ m). Consistent with the increase in PI hydrolysis and MLC₂₀ phosphorylation, treatment of freshly dispersed muscle cell with CCh (0.1 μ M) induced muscle contraction (29 \pm 3% decrease in muscle cell length). Carbachol also caused muscle contraction in cells treated with M β CD (14 \pm 1% decrease in cell length); however, contraction was significantly attenuated (51 \pm 4% inhibition, $p < 0.01$) compared to CCh response in control cells (Fig. 14). M β CD induced decrease in muscle contraction is consistent with its inhibitory effect on CCh-induced PI hydrolysis and MLC₂₀ phosphorylation in dispersed muscle cells. The specific involvement of caveolin-1 in CCh-induced muscle contraction was examined

using caveolin-1 KO mice. Basal lengths of gastric muscle cells isolated from WT mice were similar to that of caveolin-1 KO mice (WT: 92 ± 5 μm ; KO: 98 ± 4 μm). Carbachol (0.1 μM) caused a significant contraction ($29 \pm 2\%$ decrease in muscle cell length) in gastric muscle cells isolated from WT mice that was similar to contraction in isolated rabbit gastric muscle cells ($29 \pm 3\%$ decrease in cell length). Carbachol (0.1 μM) also caused muscle contraction in muscle cells isolated from caveolin-1 KO mice ($16 \pm 2\%$ decrease in muscle cell length), however, contraction was significantly attenuated ($44 \pm 4\%$ inhibition, $p < 0.01$) compared to CCh response in WT mice, and the attenuation was similar to that obtained in rabbit gastric muscle cells treated with M β CD, suggesting that caveolin-1 positively regulates initial Ca^{2+} -dependent muscle contraction (Fig. 14).

In summary, stimulation of PI hydrolysis, MLC₂₀ phosphorylation and muscle contraction in response to CCh was attenuated in dispersed cells treated with M β CD or in cultured cells transfected with caveolin-1 siRNA. Similar inhibition of PI hydrolysis and muscle contraction was obtained in gastric muscle cells from caveolin-1 KO mice compared to gastric muscle cells of WT mice. These results suggest that caveolin-1 positively regulates G_q-coupled m3 receptor signaling.

Fig. 9. Expression of caveolin-1 and -3 in gastric smooth muscle.

Total RNA was isolated from cultured gastric smooth muscle cells (first passage) using RNAqueous kit (Ambion, Austin, TX). The RNA was subsequently treated with DNAase and reverse transcribed using 2µg of total RNA by qScript cDNA prep kits (Quanta, Gaithersburg, MD). The cDNA prepared was used for quantitative real-time PCR (qRT-PCR) to measure real-time expression levels of caveolin-1 and 3 transcripts. For each cDNA sample, a 20 µl reaction volume was prepared using Quantitect™ SYBRgreen PCR mastermix (Qiagen, Mississauga, ON). Real-time PCR reaction mixtures were prepared in triplicates. Each primer set yielded only one PCR product whose identity and integrity was confirmed by sequence analysis of the individual bands. Standard curves were generated from a dilution series of cDNA and the data was quantified using $2^{\Delta\Delta Ct}$ method based on GAPDH amplification. The GAPDH thresholds remain constant. Relative quantification of a target gene in relation to a reference gene was calculated on the basis of delta delta CT values. Results demonstrated that caveolin-1 expression was 15 folds higher than caveolin-3 isoform. Values are means \pm S.E of three experiments.

Caveolin-1 and Caveolin-3 expression

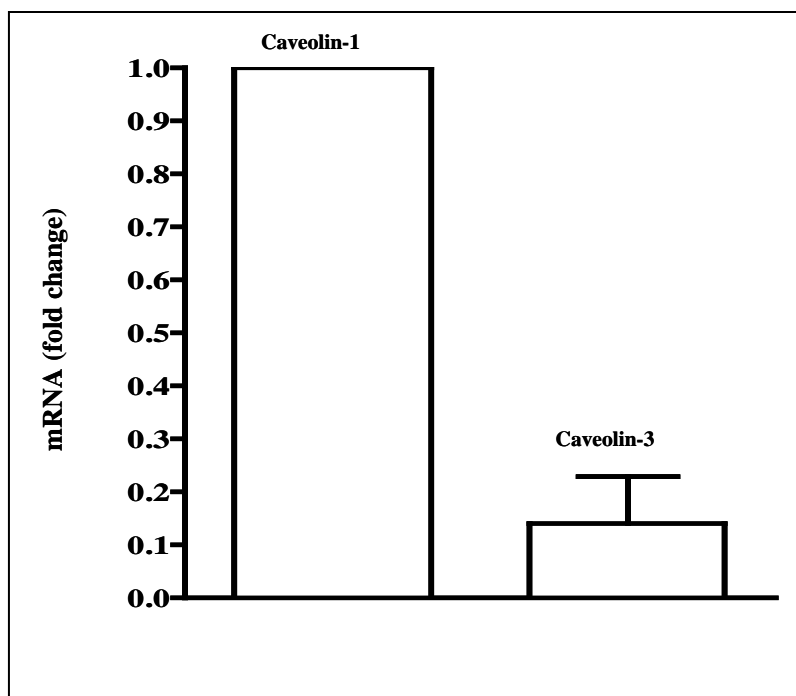


Fig.10. Inhibition of carbachol-stimulated PI hydrolysis by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were labelled with [^3H]myo-inositol and divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with CCh (0.1 μM) plus methoctramine (0.1 μM) for 60 s. In some experiments cells were pretreated with a selective PI hydrolysis inhibitor U73122 (1 μM) for 10 min and then with CCh plus methoctramine. PI hydrolysis (formation of water soluble [^3H]inositols) was measured as formation of water soluble [^3H]inositol phosphates by ion exchange chromatography and the radioactivity counted by liquid scintillation. Results are expressed as cpm/mg protein. Values are means \pm S.E of three experiments. ## Significant inhibition of CCh response in M β CD treated cells compared to CCh response in control cells ($P < 0.01$).

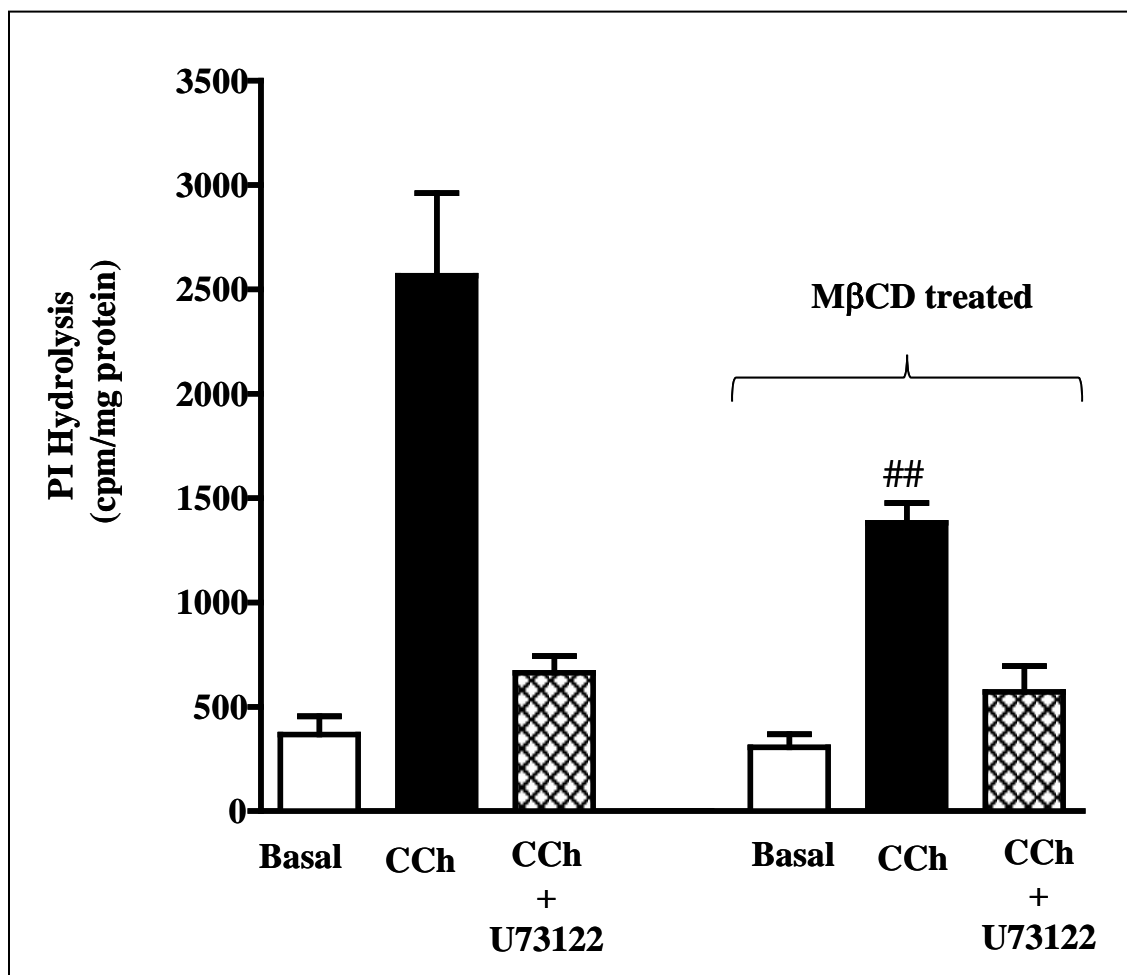


Fig. 11. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. After plasmid transfection, cultured cells were labeled with [3 H]myo-inositol, and then treated with CCh (0.1 μ M) plus methoctramine (0.1 μ M) for 60 s. In some experiments, cell were pretreated with a selective PI hydrolysis inhibitor U73122 (1 μ M) for 10 min and then treated with CCh plus methoctramine. PI hydrolysis (formation of water soluble [3 H] inositols) was measured as formation of water soluble [3 H]inositol phosphates by ion exchange chromatography and the radioactivity counted by liquid scintillation. Results are expressed as cpm/mg protein. Values are means \pm S.E of three experiments. ## Significant inhibition of CCh response in cells transfected with caveolin-1 siRNA compared to CCh response in cells transfected with vector alone ($P < 0.01$). Inset showing transfection of siRNA verified by immunofluorescence and knockdown of caveolin-1 expression by caveolin-1 siRNA verified by western blot analysis.

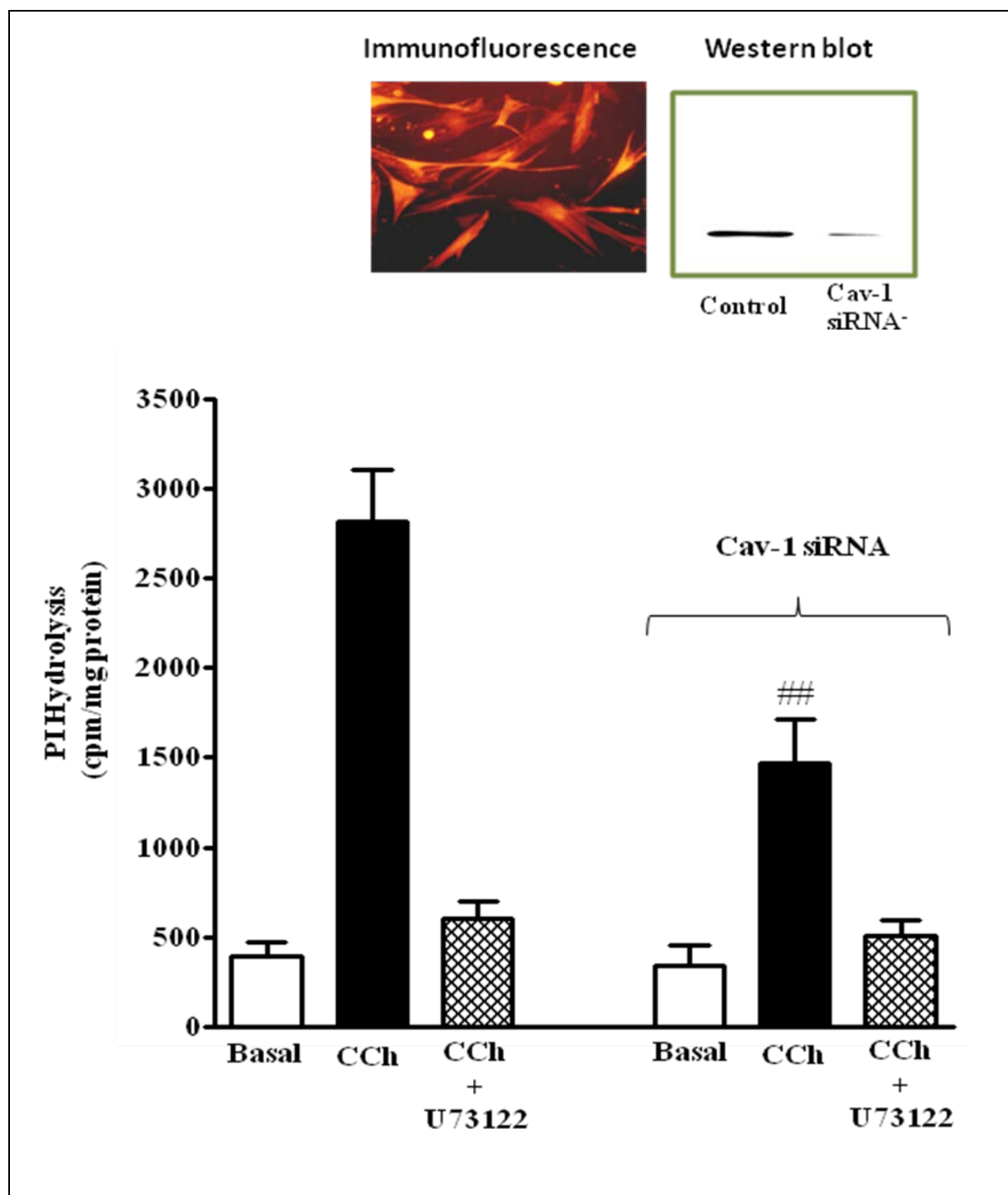


Fig.12. Inhibition of carbachol-stimulated PI hydrolysis by caveolin-1 KO.

Gastric muscle isolated from wild type and caveolin-1 KO mice were labelled with [³H]myo-inositol and then treated with CCh (0.1 μM) plus methoctramine (0.1 μM) for 60 s. In some experiments cell were pretreated with a selective PI hydrolysis inhibitor U73122 (1μM) for 10 min and then treated with CCh plus methoctramine. PI hydrolysis (formation of water soluble [³H] inositols) was measured as formation of water soluble [³H]inositol phosphates by ion exchange chromatography and the radioactivity counted by liquid scintillation. Results are expressed as cpm/mg protein. Values are means ± S.E of three experiments. ## Significant inhibition of CCh response in caveolin-1 KO mice compared to CCh response in wild type mice (P<0.01).

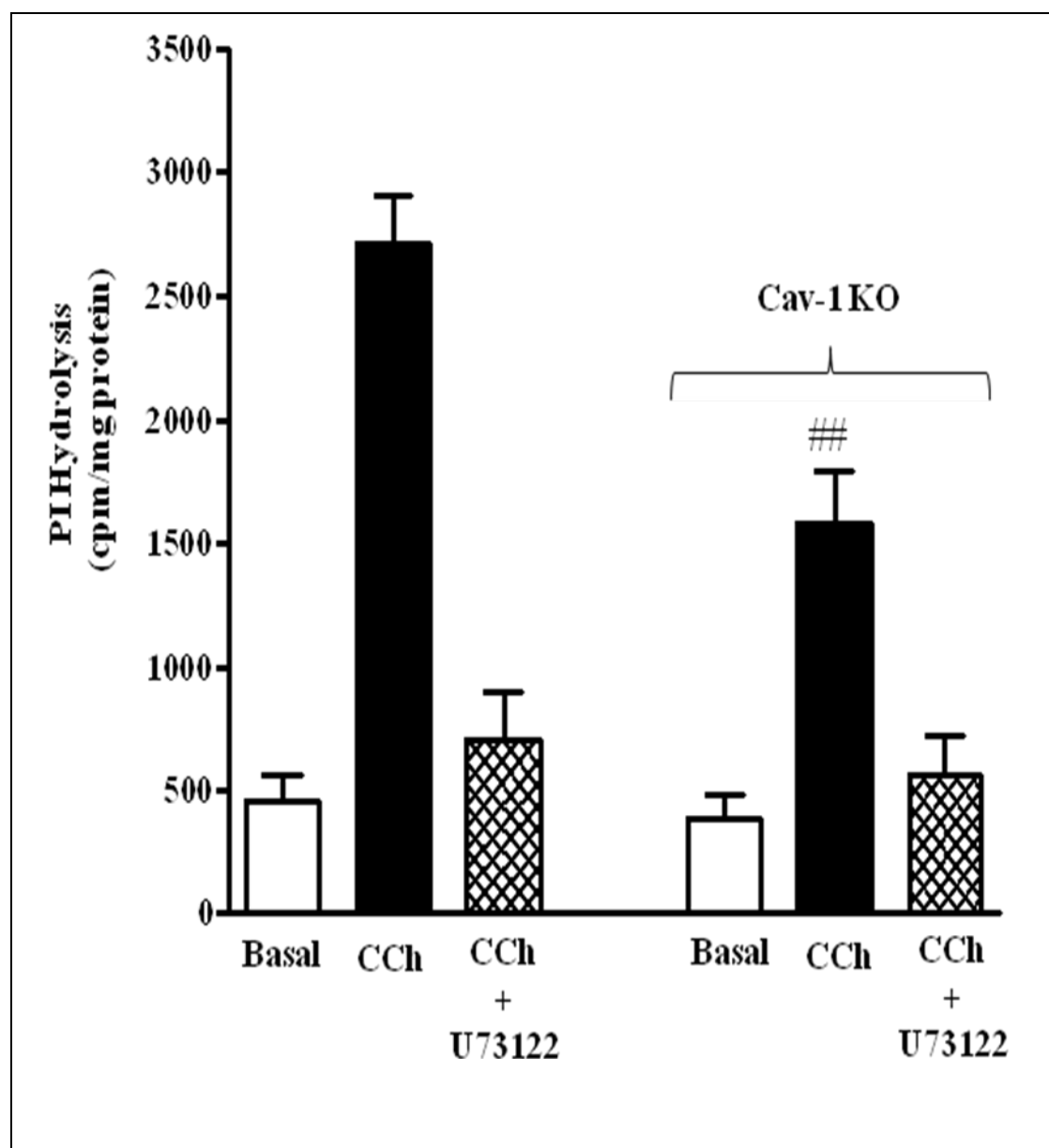


Fig. 13. Inhibition of carbachol-stimulated initial MLC₂₀ phosphorylation by MβCD

Primary culture of gastric smooth muscle cells was grown on a 96 well plate and divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β-cyclodextrin (MβCD) (10 mM) each for 30 min. After 30 min, wells in each group were treated with CCh (0.1μM) plus methoctramine (0.1μM) for 30s. MLC₂₀ phosphorylation was measured by in-cell western using phospho-specific (Ser¹⁹) antibody. Results are expressed as percent phosphorylation. Values are means ± S.E of three experiments. ** Significant inhibition of CCh response in MβCD treated cells compared to CCh response in control cells (P<0.05).

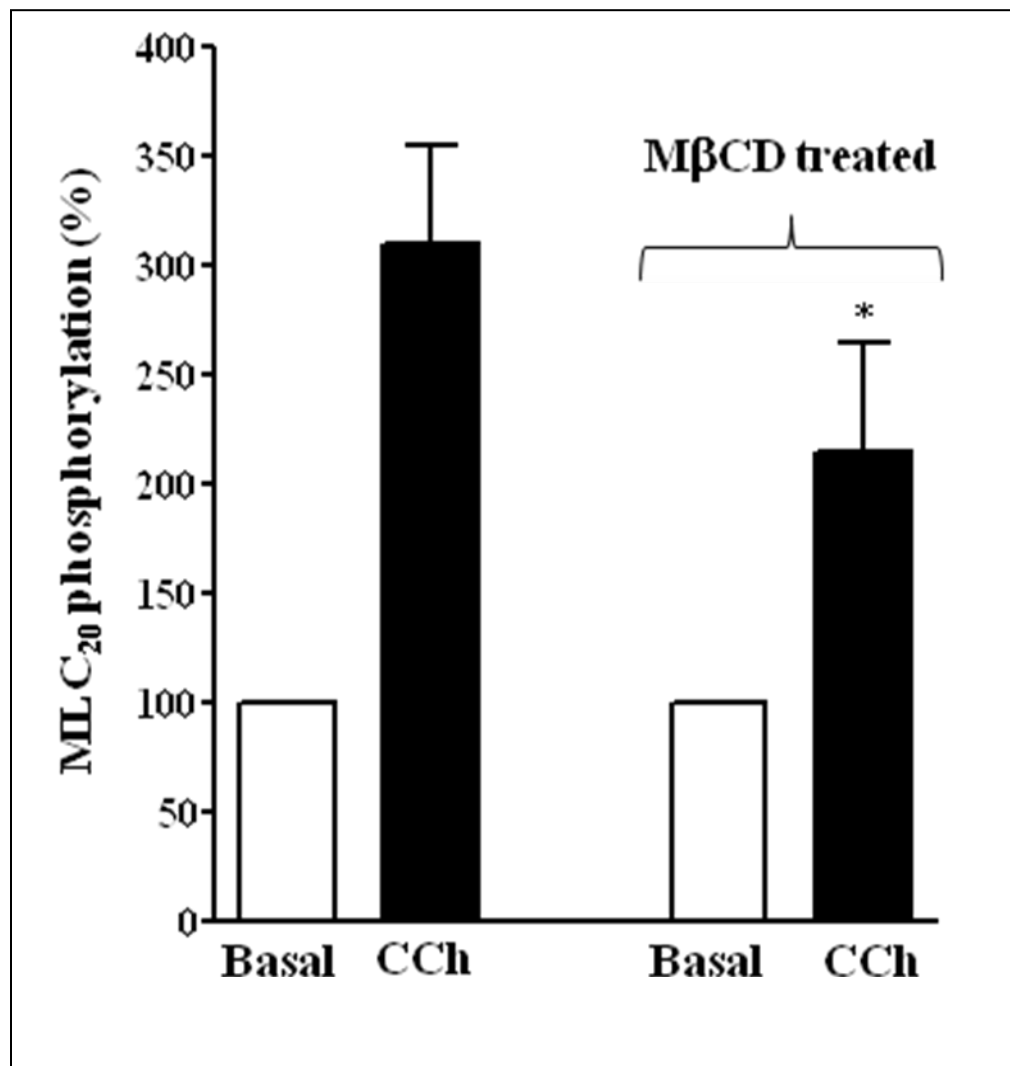
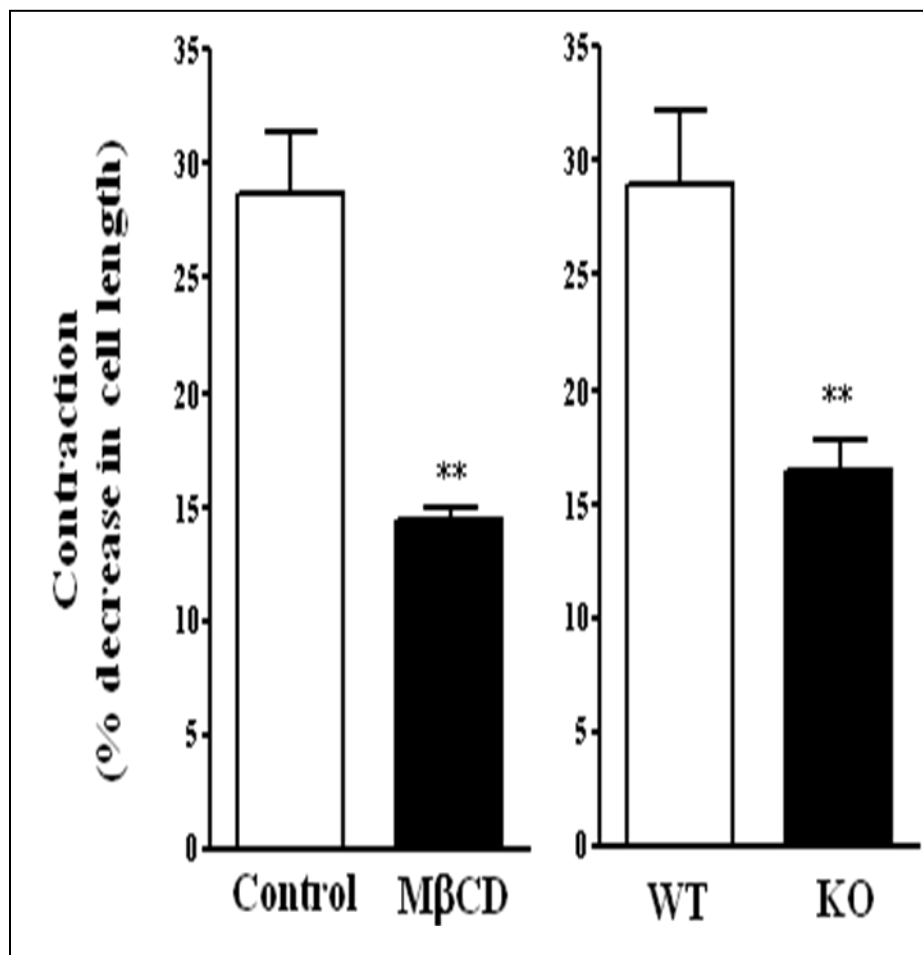


Fig. 14. Inhibition of carbachol-stimulated initial muscle contraction by M β CD and caveolin-1 KO.

Left panel: Freshly dispersed smooth muscle cells from rabbit stomach were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with CCh (0.1 μ M) plus methoctramine (0.1 μ M) for 30 s.

Right panel: Gastric muscle cells were isolated from caveolin-1 KO mice and WT mice were separately treated with CCh (0.1 μ M) plus methoctramine (0.1 μ M) for 30 s.

The mean length of 50 muscle cells was measured by scanning micrometry and compared with the length of untreated muscle cells. Results were expressed as percent decrease in cell length from control length (rabbit stomach: control (115 \pm 4 μ m) and M β CD treated (111 \pm 5 μ m); mouse stomach: wild type (92 \pm 5 μ m) and caveolin-1 KO (98 \pm 4 μ m)). Values are means \pm S.E of 5-6 experiments. ** Significant inhibition of CCh response in M β CD treated cells and in muscle cells isolated from caveolin-1 KO mice compared to CCh response in DMSO treated cells and muscle cells isolated from wild type mice (P<0.05).



III. 4. Regulation of G₁₃-coupled m3 receptor signaling by caveolae and caveolin-1.

Previous studies have demonstrated that muscarinic m3 receptors are coupled to stimulation of Rho kinase and ZIP kinase activity via G₁₃/RhoA (Murthy, Zhou et al. 2003). Stimulation of Rho kinase and ZIP kinase activity in response to m3 receptor activation was confirmed in gastric muscle cells in the present study. To examine whether caveolae and caveolin-1 also regulated G₁₃/RhoA pathway, stimulation of Rho kinase and ZIP kinase activity in response to CCh was measured in freshly dispersed muscle cells treated in the presence or absence of MβCD, cultured muscle cells transfected with control siRNA and caveolin-1 siRNA, and in gastric muscle from WT and caveolin-1 KO mice.

III. 4a. Inhibition of carbachol-stimulated Rho kinase activity by methyl β-cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β-cyclodextrin (MβCD) (10 mM) each for 30 min. After 30 min, cells were treated with CCh (0.1 μM) for 10 min, and Rho kinase activity was measured by immunokinase assay as described in Methods. The basal levels of Rho kinase activity in both groups are similar (8,411±1584 cpm/mg protein in control cells vs. 8,431±1600 cpm/mg protein in cells treated with MβCD). Treatment of control cells with CCh (0.1 μM) caused a significant increase in Rho kinase activity (33,505 cpm/mg protein; 298±18% increase above basal, p<0.001). Carbachol also caused an increase in Rho kinase activity in cells treated with MβCD (19,570±2039 cpm/mg protein; 132±6% increase above basal, p<0.001), however, the increase was significantly attenuated (54±6% inhibition, p<0.001) compared to CCh response in control cells (Fig. 15). The selective Rho kinase inhibitor, Y27632 (1 μM) blocked CCh-stimulated Rho kinase activity in

both control cells and in cells treated with M β CD (Fig. 15). These results suggest that caveolae positively regulates G₁₃/RhoA-mediated Rho kinase activity in gastric muscle cells.

III . 4b. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Suppression of caveolin-1 expression was verified by western blot analysis and immunofluorescence. Muscle cells were treated with CCh (0.1 μ M) for 10 min, and Rho kinase activity was measured by immunokinase assay as described in Methods. The basal levels of Rho kinase activity in both groups are similar (7,582 \pm 1217 cpm/mg protein in control cells vs. 8,126 \pm 1906 cpm/mg protein in cells transfected with caveolin-1 siRNA). Treatment of control cells with CCh caused a significant increase in Rho kinase activity (33,288 \pm 4549 cpm/mg protein; 239 \pm 8% increase above basal, p <0.001). Carbachol (0.1 μ M) also caused an increase in Rho kinase activity in cells transfected with caveolin-1 siRNA (21,579 \pm 3842 cpm/mg protein; 165 \pm 6% increase above basal, p <0.001); however, the increase was significantly attenuated (31 \pm 4 % inhibition, p <0.01) compared to CCh response in control cells (Fig. 16). The selective Rho kinase inhibitor, Y27632 (1 μ M), blocked CCh-stimulated Rho kinase activity in both control cells and in cells transfected with caveolin-1 siRNA (Fig. 16). These results suggest that caveolin-1 positively regulates G₁₃/RhoA-mediated Rho kinase activity in gastric muscle cells.

III . 4c. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 KO.

Gastric muscle was isolated from wild type and caveolin-1 knockout mice and Rho kinase activity in response to CCh (0.1 μ M) was measured by immunokinase assay. The basal

levels of Rho kinase activity in gastric muscle from control and caveolin-1 KO mice were similar (7460 ± 1895 cpm/mg protein in WT mice vs. $7,150 \pm 1338$ cpm/mg protein in caveolin-1 KO mice). Treatment of muscle from WT mice with CCh ($0.1 \mu\text{M}$) caused a significant increase in Rho kinase activity ($30,033$ cpm/mg protein: $302 \pm 10\%$ increase above basal, $p < 0.001$). Carbachol ($0.1 \mu\text{M}$) also caused an increase in Rho kinase activity in muscle from caveolin-1 KO mice ($19,090 \pm 2049$ cpm/mg protein; $166 \pm 5\%$ increase above basal, $p < 0.001$); however, the increase was significantly attenuated ($45 \pm 8\%$ inhibition, $p < 0.01$) compared to CCh response in WT mice (Fig. 17). The selective Rho kinase inhibitor, Y27632 ($1 \mu\text{M}$), blocked CCh-stimulated Rho kinase activity in both WT and caveolin-1 KO mice (Fig.17). These results provide conclusive evidence that caveolin-1 positively regulates G_{13} /RhoA-mediated Rho kinase activity in gastric muscle.

III . 4d. Inhibition of carbachol-stimulated ZIP kinase activity by methyl β -cyclodextrin.

Freshly dispersed muscle cells incubated in the presence or absence of M β CD (10 mM) for 30 min were treated with CCh ($0.1 \mu\text{M}$) for 10 min, and ZIP kinase activity was measured by immunokinase assay as described in Methods. The basal level of ZIP kinase activity was similar in the presence or absence of M β CD ($4,058 \pm 921$ cpm/mg protein in control cells vs. $4,039 \pm 975$ cpm/mg protein in cells treated with M β CD). Treatment of control cells with CCh ($0.1 \mu\text{M}$) caused a significant increase in ZIP kinase activity ($19,726 \pm 2213$ cpm/mg protein; $386 \pm 11\%$ increase above basal, $p < 0.001$). Carbachol ($0.1 \mu\text{M}$) also caused an increase in ZIP kinase activity in cells treated with M β CD ($12,028 \pm 2013$ cpm/mg protein; $197 \pm 12\%$ increase above basal, $p < 0.001$), however, the increase was significantly attenuated ($48 \pm 6\%$ inhibition, $p < 0.01$) compared to CCh response in control cells (Fig. 18). The selective Rho kinase inhibitor, Y27632

(0.1 μ M) blocked CCh-stimulated ZIP kinase activity in both control cells and in cells treated with M β CD, suggesting that ZIP kinase activity is downstream of Rho kinase activity (Fig. 18). These results suggest that caveolae positively regulates G₁₃/RhoA-mediated ZIP kinase activity in gastric muscle cells.

III . 4e. Inhibition of carbachol-stimulated ZIP kinase activity by caveolin-1 siRNA.

Muscle cells transfected with control siRNA or caveolin-1 siRNA were treated with CCh (0.1 μ M) for 10 min, and ZIP kinase activity was measured by immunokinase assay as described in Methods. The basal level of ZIP kinase activity was not affected by the transfection of caveolin-1 siRNA (3,609 \pm 1016 cpm/mg protein in control cell vs. 4,206 \pm 1127 cpm/mg protein in cells transfected with caveolin-1 siRNA). Treatment of control cells with CCh (0.1 μ M) caused a significant increase in ZIP kinase activity (21,414 \pm 3043 cpm/mg protein; 493 \pm 25% increase above basal, p <0.001). Carbachol (0.1 μ M) also caused an increase in ZIP kinase activity in cells treated with caveolin-1 siRNA (12,507 \pm 2201 cpm/mg protein; 194 \pm 21% increase above basal, p <0.001), however, the increase was significantly attenuated (59 \pm 6% inhibition, p <0.01) compared to CCh response in control cells (Fig. 19). Y27632 (1 μ M) blocked CCh-stimulated ZIP kinase activity in both control cells and in cells transfected with caveolin-1 siRNA. These results suggest that caveolin-1 positively regulates G₁₃/RhoA-mediated ZIP kinase activity in gastric muscle cells.

III. 4f. Inhibition of carbachol-stimulated ZIP kinase activity by caveolin-1 KO.

Gastric muscle was isolated from wild type and caveolin-1 KO mice and ZIP kinase activity in response to CCh (0.1 μ M) was measured by immunokinase assay. The basal levels of

ZIP kinase activity in gastric muscle from control and caveolin-1 KO mice were similar ($3,136 \pm 644$ cpm/mg protein in WT mice vs. $3,515 \pm 755$ cpm/mg in caveolin-1 KO mice). Treatment of muscle from WT mice with CCh ($0.1 \mu\text{M}$) caused a significant increase in ZIP kinase activity ($18,003 \pm 2941$ cpm/mg protein: $474 \pm 17\%$ increase above basal, $p < 0.001$). Carbachol ($0.1 \mu\text{M}$) also caused an increase in ZIP kinase activity in muscle from caveolin-1 KO mice ($11,213 \pm 1701$ cpm/mg protein; $219 \pm 16\%$ increase above basal, $p < 0.001$); however, the increase was significantly attenuated ($54 \pm 6\%$ inhibition, $p < 0.01$) compared to CCh response in WT mice (Fig. 20). Y27632 ($1 \mu\text{M}$) blocked CCh-stimulated Rho kinase activity in both WT and caveolin-1 KO mice (Fig. 20). These results provide conclusive evidence that caveolin-1 positively regulates G_{13} /RhoA-mediated ZIP kinase activity in gastric muscle.

III. 4g. Inhibition of carbachol-stimulated MYPT1 phosphorylation and sustained muscle contraction by M β CD and caveolin-1 KO.

Previous studies in gastrointestinal muscle have demonstrated that sustained contraction is mediated by stimulation of Rho-dependent pathways involving phosphorylation of MYPT1 (regulatory subunit of MLC phosphatase) at Thr⁶⁹⁶ by Rho kinase/ZIP kinase and CPI-17 (endogenous MLC phosphatase inhibitor) at Thr³⁸ by PKC. Phosphorylation of MYPT1 and CPI-17 leads to inhibition of MLC phosphatase activity, increase in MLC₂₀ phosphorylation and sustained muscle contraction (Murthy, Grider et al. 2000). Since there is decrease in Rho kinase/ZIP kinase activity by disruption of caveolae with M β CD, we tested the hypothesis that MYPT1 phosphorylation and sustained muscle contraction are positively regulated by caveolin-1. The effect of M β CD (10 mM) on CCh-induced MYPT1 phosphorylation and contraction in freshly dispersed muscle cells was examined. MYPT1 phosphorylation was measured by in-cell

western using phospho-specific (Thr⁶⁹⁶) antibody. Treatment of cells with CCh (0.1 μ M) for 10 min significantly increased MYPT1 phosphorylation ($314 \pm 43\%$ increase, $p < 0.001$). Carbachol also caused an increase in MYPT1 phosphorylation in cells treated with M β CD ($126 \pm 15\%$ increase, $p < 0.001$); however, the increase was significantly attenuated ($58 \pm 10\%$ inhibition, $p < 0.01$) compared to CCh response in control cells (Fig. 21).

Treatment of cells with M β CD (10 mM) had no significant effect on basal muscle cell length (control: 115 ± 4 μ m vs. M β CD treated: 111 ± 5 μ m). Consistent with the increase in Rho kinase and ZIP kinase activity and MYPT1 phosphorylation, treatment of freshly dispersed muscle cell with CCh (0.1 μ M) induced sustained (measured at 10 min) muscle contraction ($22 \pm 3\%$ decrease in muscle cell length). Carbachol also caused muscle contraction in cells treated with M β CD ($11 \pm 2\%$ decrease in cell length); however, contraction was significantly attenuated ($49 \pm 3\%$ inhibition, $p < 0.01$) compared to CCh response in control cells (Fig. 22). M β CD-induced decrease in muscle contraction is consistent with its inhibitory effect on CCh-induced Rho kinase/ZIP kinase activity and MYPT1 phosphorylation in dispersed muscle cells.

The specific involvement of caveolin-1 in CCh-induced sustained muscle contraction was examined using caveolin-1 KO mice. Basal lengths of gastric muscle cells isolated from WT mice were similar to that of caveolin-1 KO mice (WT: 92 ± 5 μ m; KO: 98 ± 4 μ m). Carbachol (0.1 μ M) caused a significant contraction ($21 \pm 2\%$ decrease in muscle cell length) in gastric muscle cells isolated from WT mice that was similar to the contraction in isolated rabbit gastric muscle cells ($22 \pm 3\%$ decrease in cell length). Carbachol (0.1 μ M) also caused muscle contraction in muscle cells isolated from caveolin-1 KO mice ($13 \pm 2\%$ decrease in muscle cell length), however, contraction was significantly attenuated (39 ± 3 inhibition, $p < 0.01$) compared to CCh response in WT mice, and the attenuation was similar to that obtained in rabbit gastric

muscle cells treated with M β CD (Fig. 22), suggesting that caveolin-1 positively regulates RhoA-dependent sustained contraction in gastric muscle.

In summary, stimulation of Rho kinase and ZIP kinase activity, MYPT1 phosphorylation and muscle contraction in response to CCh was attenuated in dispersed cells treated with M β CD or in cultured cells transfected with caveolin-1 siRNA. Similar inhibition of Rho kinase and ZIP kinase activity and muscle contraction was obtained in gastric muscle cells from caveolin-1 KO mice compared to gastric muscle cells of WT mice. These results suggest that caveolin-1 positively regulates G₁₃-coupled m3 receptor signaling.

Fig. 15. Inhibition of carbachol-stimulated Rho kinase activity by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with CCh (0.1 μ M) for 10 min in the presence or absence of a selective Rho kinase inhibitor Y27632 (1 μ M). Rho kinase activity was measured using [γ - 32 P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values are means \pm S.E of 6 experiments. ## Significant inhibition of CCh response in M β CD treated cells compared to CCh response in control cells (P<0.01).

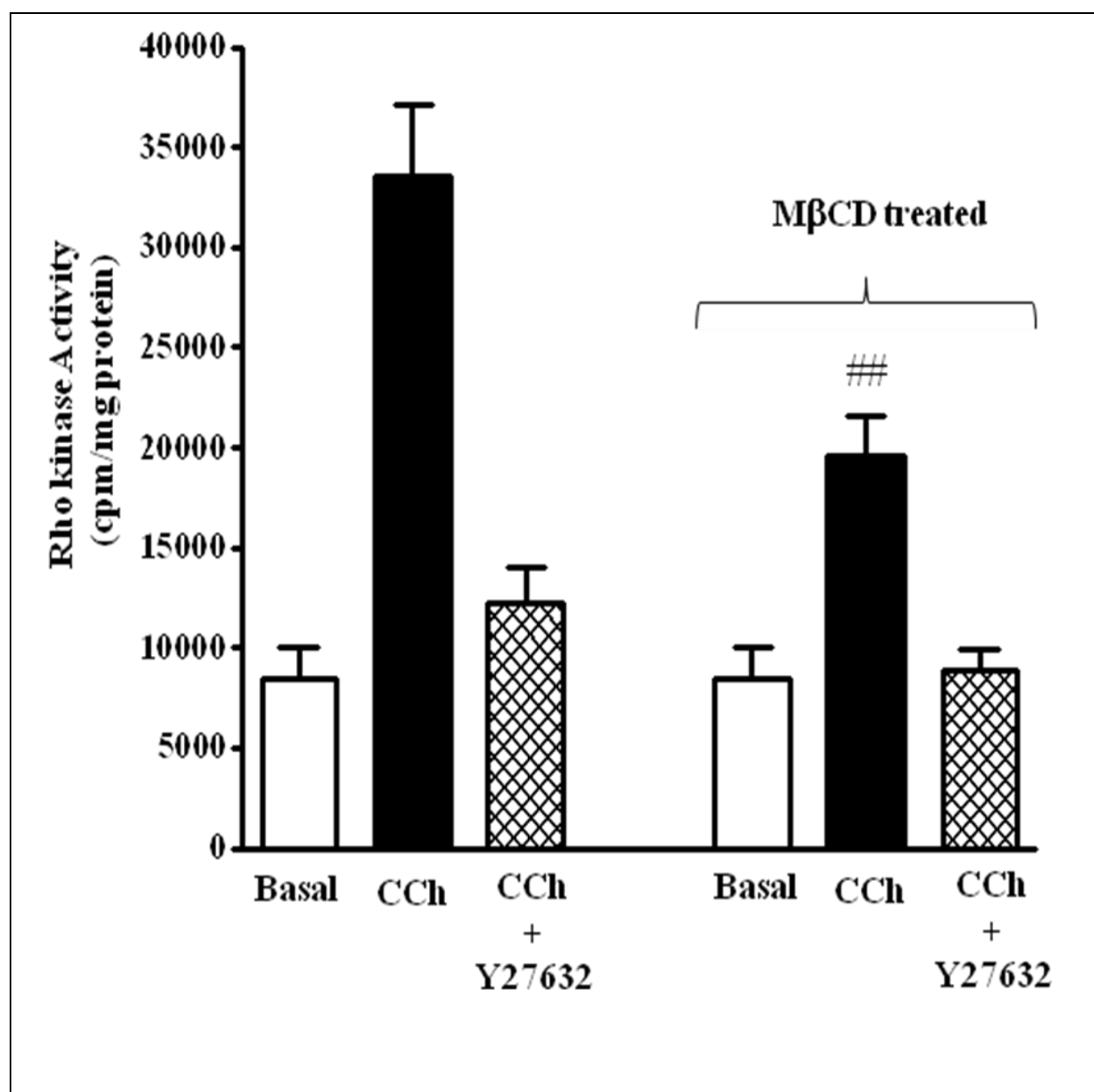


Fig. 16. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. After plasmid transfection, cells were treated with CCh (0.1 μ M) for 10 min in the presence or absence of a selective Rho kinase inhibitor, Y27632. Rho kinase activity was measured using [γ - 32 P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values are means \pm S.E of 4 experiments. ## Significant inhibition of CCh response in cells transfected with caveolin-1 siRNA compared to CCh response in cells transfected with control pSIREN-DNR-DsRed vector (P<0.01).

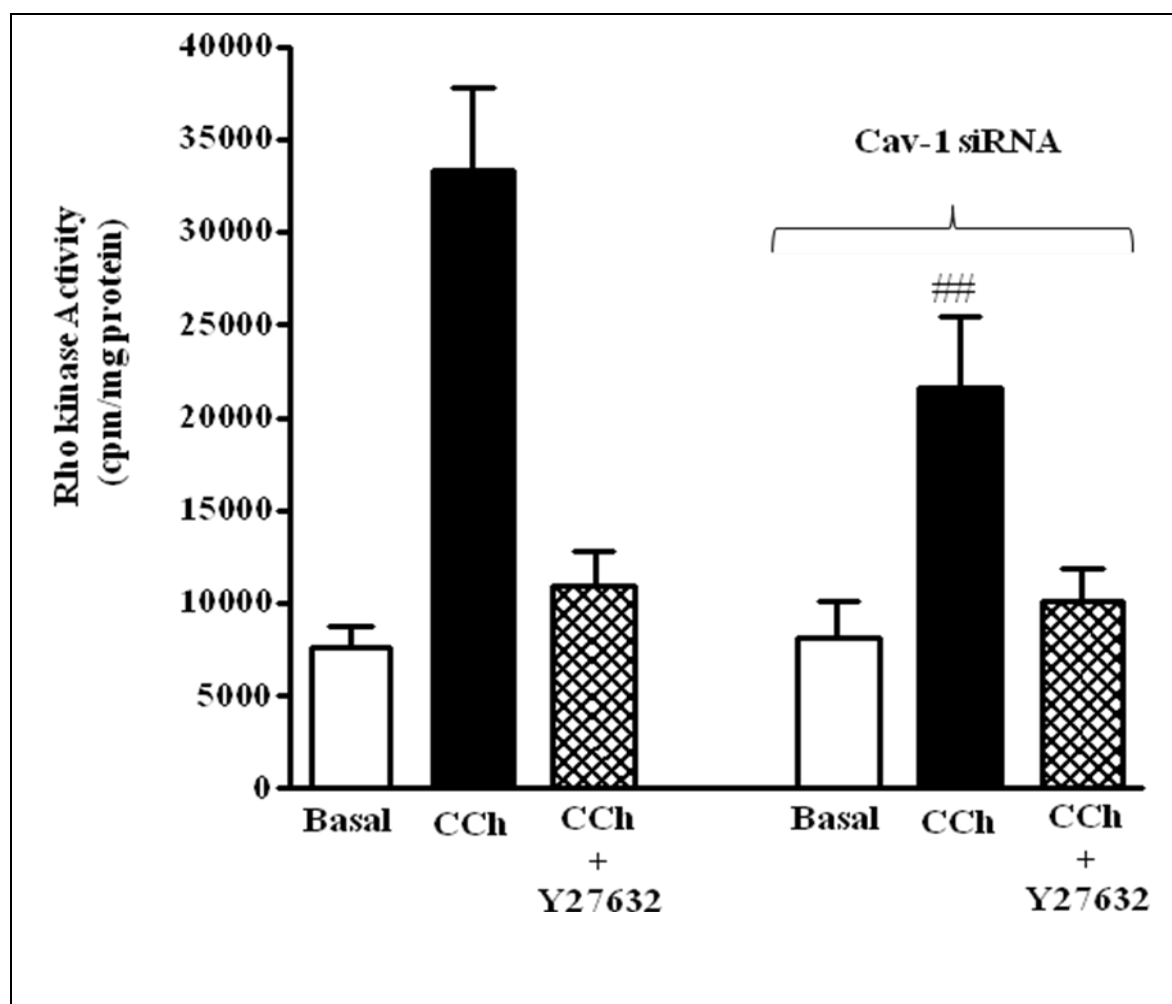


Fig. 17. Inhibition of carbachol-stimulated Rho kinase activity by caveolin-1 KO.

Gastric muscle isolated from wild type and caveolin-1 KO mice were treated with CCh (0.1 μ M) for 10 min in the presence or absence of Y27632 (1 μ M). Rho kinase activity was measured using [γ - 32 P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values are means \pm S.E of 3 experiments. ## Significant inhibition of CCh response in caveolin-1 KO mice compared to CCh response in wild type mice (P<0.01).

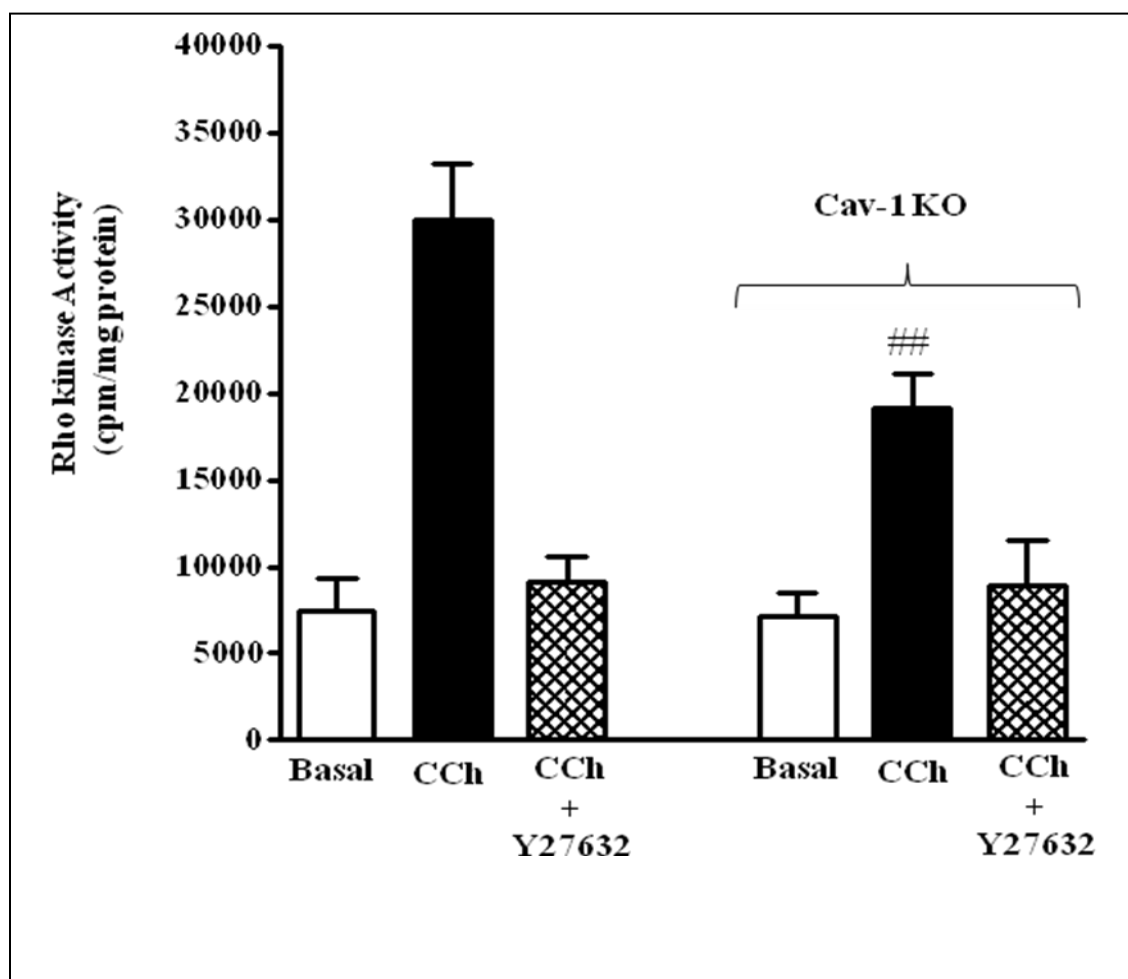


Fig. 18. Inhibition of carbachol-stimulated zipper interacting protein (ZIP) kinase activity by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with CCh (0.1 μ M) for 10 min in the presence or absence of a selective Rho kinase inhibitor Y27632 (1 μ M). ZIP kinase activity was measured using [γ - 32 P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values are means \pm S.E of 6 experiments. ## Significant inhibition of CCh response in M β CD treated cells compared to CCh response in control cells (P<0.01).

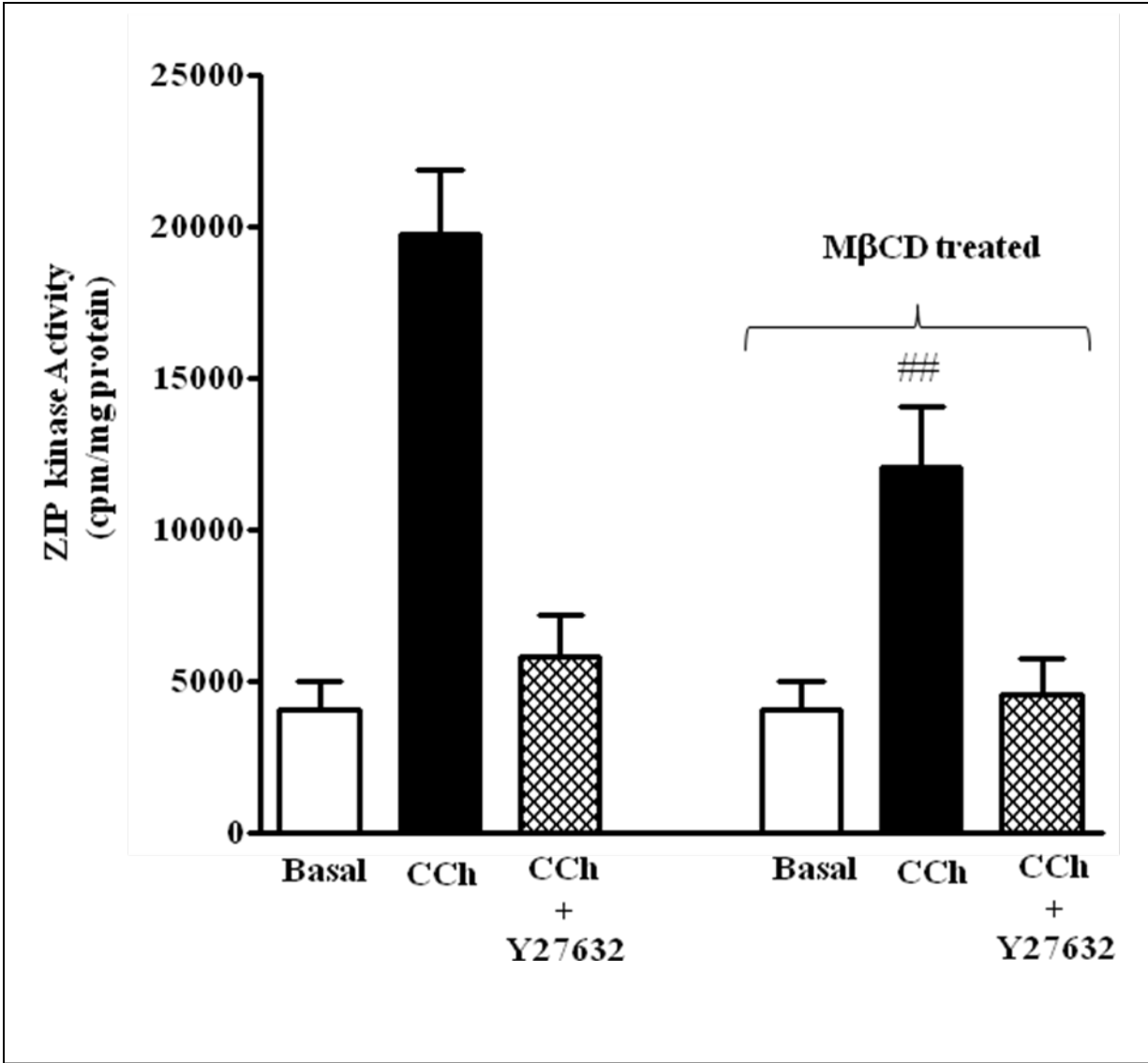


Fig. 19. Inhibition of carbachol-stimulated zipper interacting protein (ZIP) kinase activity by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. After plasmid transfection, cells were treated with CCh (0.1 μ M) for 10 min in the presence or absence of a selective Rho kinase inhibitor, Y27632. ZIP kinase activity was measured using [γ -³²P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values are means \pm S.E of 4 experiments. ## Significant inhibition of CCh response in cells transfected with caveolin-1 siRNA compared to CCh response in control cells (P<0.01).

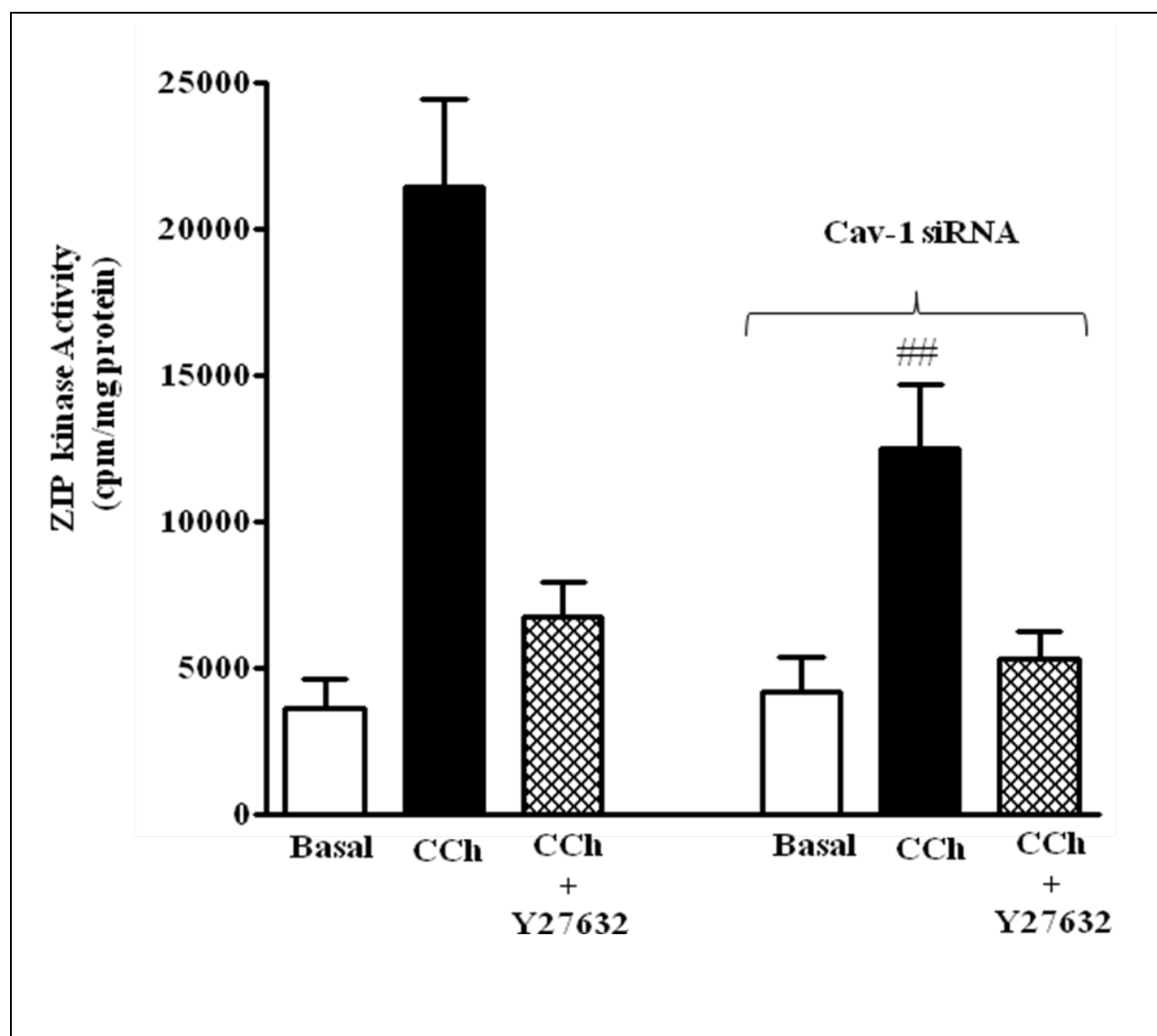


Fig. 20. Inhibition of carbachol-stimulated zipper interacting protein (ZIP) kinase activity by caveolin-1 KO.

Gastric muscle isolated from wild type and caveolin-1 KO mice were treated with CCh (0.1 μ M) for 10 min in the presence or absence of Y27632 (1 μ M). ZIP kinase activity was measured using [γ -³²P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values are means \pm S.E of 3 experiments. ## Significant inhibition of CCh response in caveolin-1 KO mice compared to CCh response in wild type mice (P<0.01).

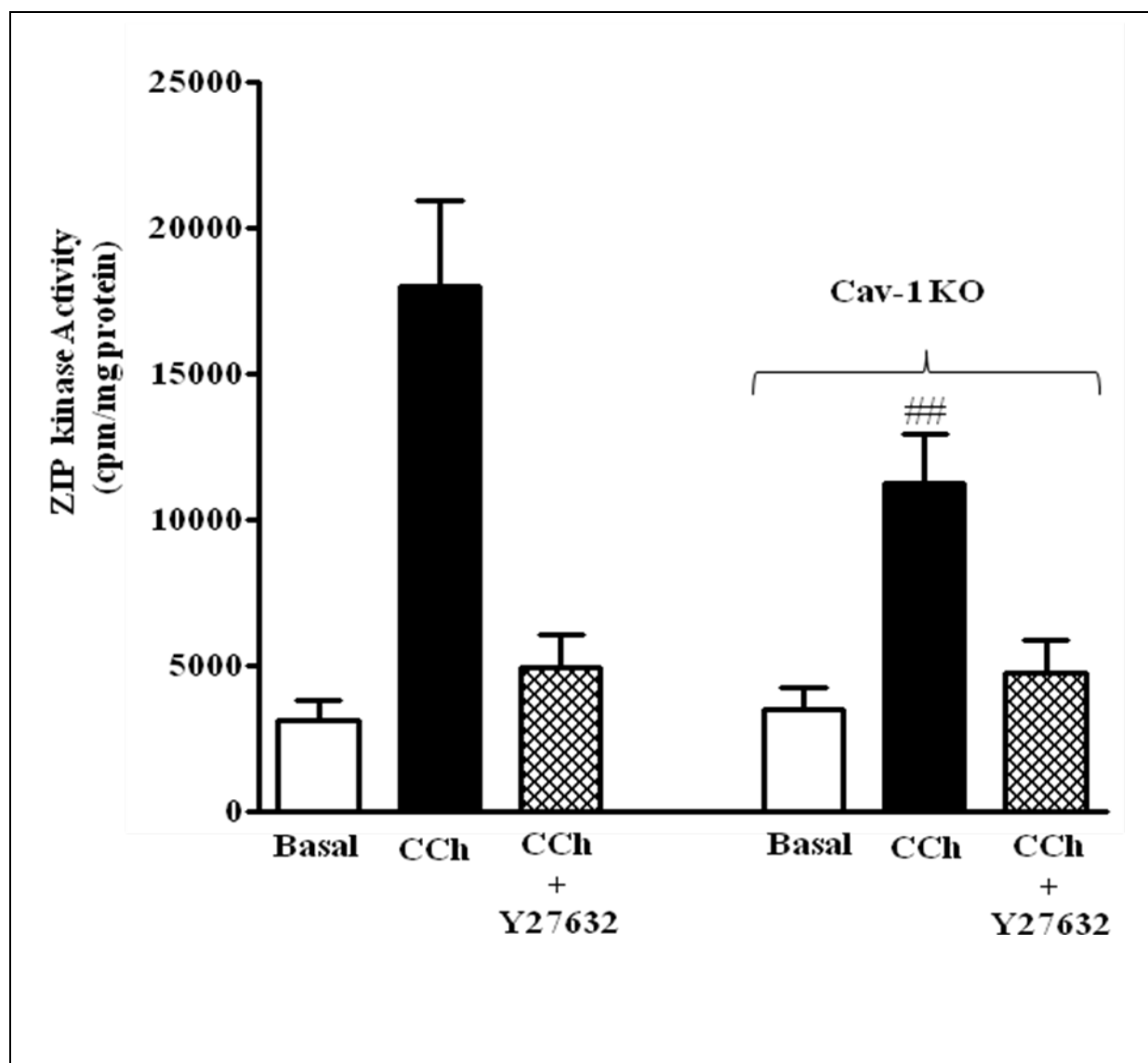


Fig. 21. Inhibition of carbachol-stimulated MYPT1 phosphorylation by M β CD

Primary culture of gastric smooth muscle cells was grown on a 96 well plate and divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, wells in each group were treated with CCh (0.1 μ M) for 10 min and MYPT1 phosphorylation was measured by in-cell western using phospho-specific (Thr⁶⁹⁶) antibody. Results are expressed as percent phosphorylation. Values are means \pm S.E of 4 experiments. ** Significant inhibition of CCh response in M β CD treated cells compared to CCh response in control cells (P<0.01).

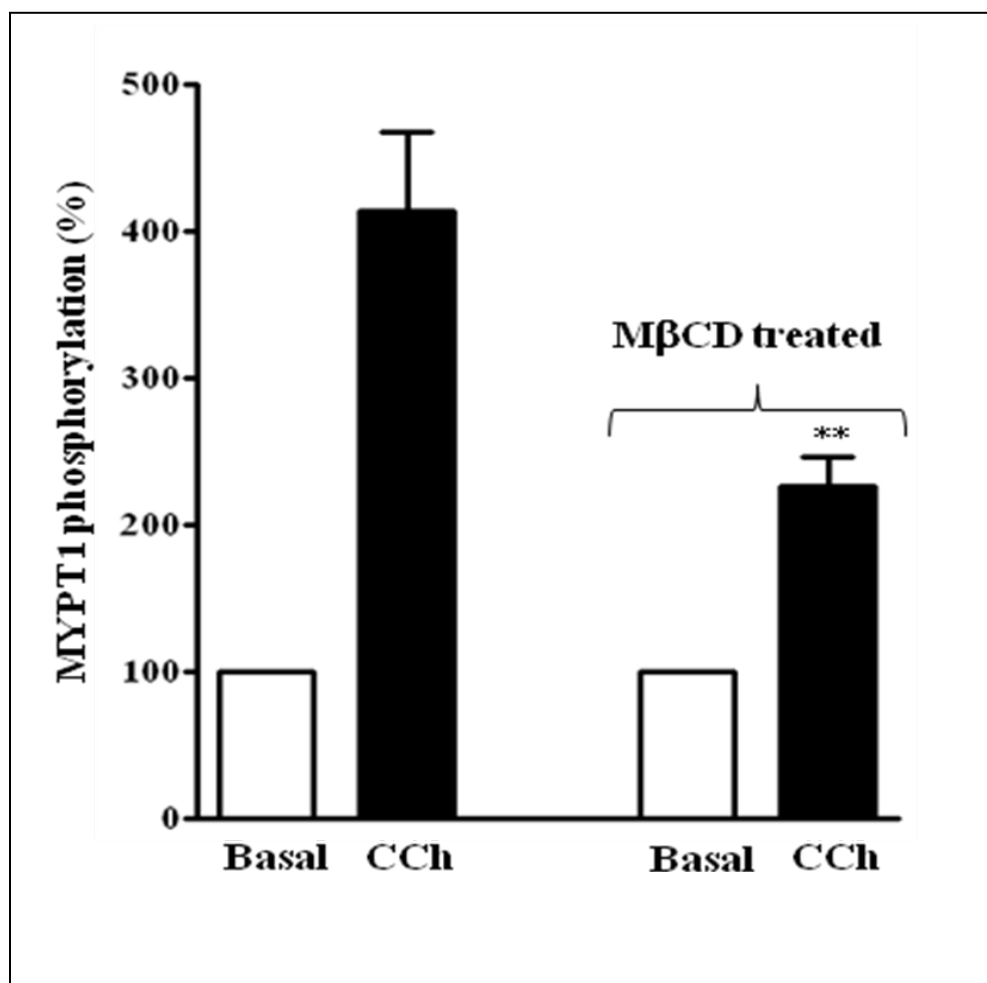
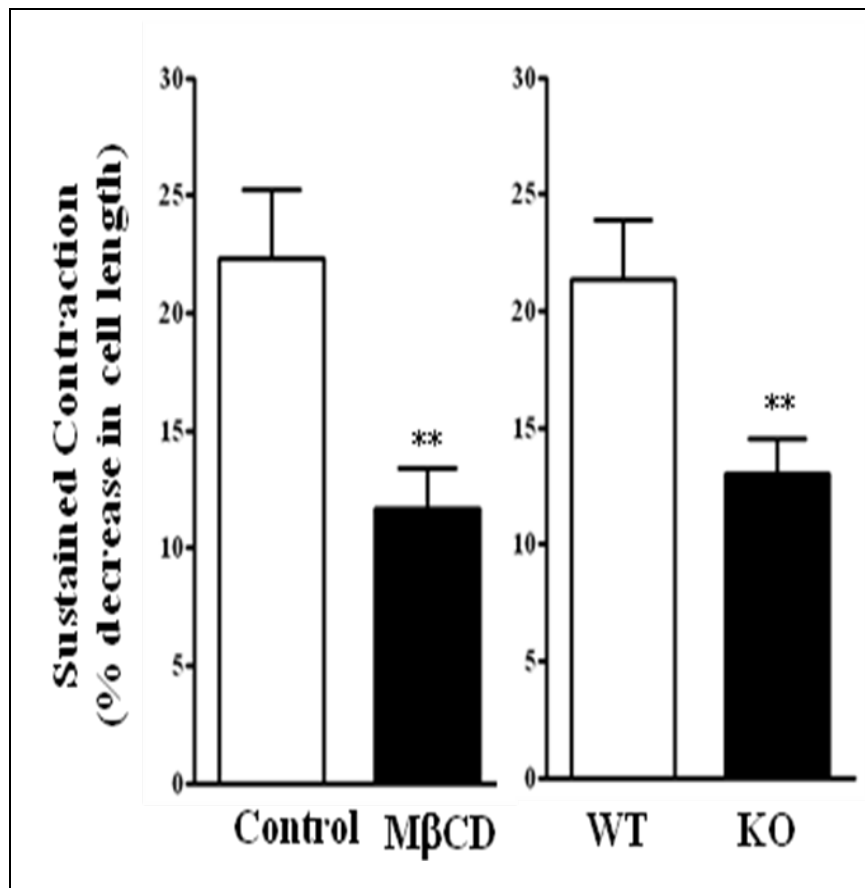


Fig. 22. Inhibition of carbachol-stimulated sustained muscle contraction by M β CD and caveolin-1 KO.

Left panel: Freshly dispersed smooth muscle cells from rabbit stomach were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with CCh (0.1 μ M) plus methoctramine (0.1 μ M) for 10 min. **Right panel:** Gastric muscle cells were isolated from caveolin-1 KO mice and WT mice were separately treated with CCh (0.1 μ M) plus methoctramine for 10 min.

The mean length of 50 muscle cells was measured by scanning micrometry and compared with the length of untreated muscle cells. Results were expressed as percent decrease in cell length from control length (rabbit stomach: control (115 \pm 4 μ m) and M β CD treated (111 \pm 5 μ m); mouse stomach: wild type (92 \pm 5 μ m) and caveolin-1 KO (98 \pm 4 μ m)). Values are means \pm S.E of 5-6 experiments. ** Significant inhibition of CCh response in M β CD treated cells and in muscle cells isolated from caveolin-1 KO mice compared to CCh response in DMSO treated cells and muscle cells from wild type mice (P<0.01).



III. 5. Regulation of G_i-coupled muscarinic m2 receptor signaling by caveolin-1.

Studies from our group and several others have demonstrated that muscarinic m2 receptors are coupled to inhibition of adenylyl cyclase activity (AC) via activation of G α_i proteins. To examine whether caveolae and caveolin-1 also regulate G_i-coupled adenylyl cyclase activity, inhibition of forskolin-stimulated cAMP formation in response to CCh was measured using three approaches mentioned above.

III. 5a. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with forskolin (10 μ M) for 10 min in the presence or absence of CCh (0.1 μ M), and adenylyl cyclase activity was measured as increase in cAMP formation by radioimmunoassay as described in Methods. The basal levels of cAMP were similar in both groups (3.9 \pm 1.2 pmol/mg protein in control cell vs. 3.8 \pm 0.8 pmol/mg protein in cells treated with M β CD). Treatment of cells with forskolin (10 μ M) caused an increase in cAMP formation and the increase was similar in both groups (531 \pm 34% increase above basal in control cells and 479 \pm 32% increase above basal in cells treated with M β CD) (Fig. 23). These results suggest that caveolae has no significant effect on adenylyl cyclase activity in gastric muscle cells. Carbachol (0.1 μ M) caused a significant inhibition of forskolin-stimulated cAMP formation in control cells (168 \pm 20% increase in cAMP above basal, and 68 \pm 5% attenuation compared to response to forskolin alone, p <0.001) and in cells treated with M β CD (142 \pm 16% increase above basal, and 71 \pm 6% attenuation compared to

forskolin alone, $p < 0.001$). The inhibition of forskolin-stimulated cAMP by CCh is similar in control cells and in cells treated with M β CD (Fig. 23), suggesting that caveolae has no effect on m2-coupled G $_i$ activity in gastric muscle cells.

III. 5b. Lack of effect on m2-mediated inhibition of adenylyl cyclase (AC) activity by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Suppression of caveolin-1 expression was verified by western blot analysis and immunofluorescence. Muscle cells were treated with forskolin (10 μ M) for 10 min in the presence or absence of CCh (0.1 μ M), and adenylyl cyclase activity was measured. The basal levels of cAMP were similar in both groups (2.7 ± 0.8 pmol/mg protein in control cell vs. 2.5 ± 0.6 pmol/mg protein in cells transfected with caveolin-1 siRNA). Treatment of cells with forskolin (10 μ M) caused an increase in cAMP formation and the increase was similar in both groups ($738 \pm 42\%$ increase above basal in control cells and $682 \pm 54\%$ increase above basal in cells transfected with caveolin-1 siRNA) (Fig. 24). These results suggest that caveolin-1 has no significant effect on adenylyl cyclase activity in gastric muscle cells. Carbachol caused a significant inhibition of forskolin-stimulated cAMP formation in control cells ($218 \pm 18\%$ increase in cAMP above basal, and $70 \pm 6\%$ attenuation compared to response to forskolin alone, $p < 0.001$) and in cells transfected with caveolin-1 siRNA ($221 \pm 19\%$ increase above basal, and $67 \pm 6\%$ attenuation compared to forskolin alone, $p < 0.001$). The inhibition of forskolin-stimulated cAMP by CCh is similar in control cells and in cells transfected with caveolin-1 siRNA (Fig.

24), also suggesting that caveolin-1 has no effect on m2-coupled G_i activity in gastric muscle cells.

III. 5c. Lack of effect on m2-mediated inhibition of adenylyl cyclase (AC) activity by caveolin-1 knockout.

Gastric muscle was isolated from wild type and caveolin-1 KO mice and inhibition of forskolin-stimulated cAMP formation and AC activity in response to CCh (0.1 μ M) was measured. The basal levels of cAMP were similar in both groups (3.7 ± 1.0 pmol/mg protein in WT mice vs. 3.9 ± 0.6 pmol/mg protein in KO mice). Treatment of cells with forskolin (10 μ M) caused an increase in cAMP formation and the increase was similar in both groups ($584 \pm 35\%$ increase above basal in WT mice and $541 \pm 27\%$ increase above basal in KO mice). These results suggest that caveolin-1 has no significant effect on adenylyl cyclase activity in gastric muscle cells. Carbachol (0.1 μ M) caused a significant inhibition of forskolin-stimulated cAMP formation in gastric cells isolated from caveolin-1 KO mice ($154 \pm 12\%$ increase in cAMP above basal, and $73 \pm 4\%$ attenuation compared to response to forskolin alone, $p < 0.001$) and in cells isolated from WT mice ($143 \pm 13\%$ increase above basal, and $72 \pm 9\%$ attenuation compared to forskolin alone, $p < 0.001$) (Fig. 25). The inhibition of forskolin-stimulated cAMP by CCh is similar in WT mice and caveolin-1 KO mice (Fig. 25), suggesting that caveolin-1 has no effect on m2-coupled G_i activity in gastric muscle cells.

In summary, activation of AC activity by forskolin or inhibition of forskolin-stimulated AC activity by CCh was not affected in dispersed cells treated with M β CD or in cultured muscle cells transfected with caveolin-1 siRNA. Similarly, activation of AC activity or inhibition of forskolin-stimulated AC activity by CCh was not affected by caveolin-1 KO. These results

suggest that caveolin-1 has no effect on G_i -coupled m2 receptor signaling. This is in contrast to the positive regulation of $G_{q/13}$ -coupled m3 receptor signaling by caveolin-1.

Fig. 23. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with methyl β -cyclodextrin (M β CD) (10 mM) each for 30 min. After 30 min, cells were treated with forskolin (10 μ M) for 10 min in the presence or absence of CCh (0.1 μ M) and adenylyl cyclase activity was measured as increase in cAMP formation by radioimmunoassay. Forskolin caused an increase in cAMP formation and the increase was similar in both groups. Carbachol caused a significant inhibition of forskolin-stimulated cAMP formation and the extent of inhibition was similar in control cells and in cells treated with M β CD. Results are expressed as pmol/mg protein. Values are means \pm S.E of 4 experiments. ** Significant inhibition of forskolin-stimulated cAMP formation by CCh in both control and M β CD treated cells ($P < 0.001$).

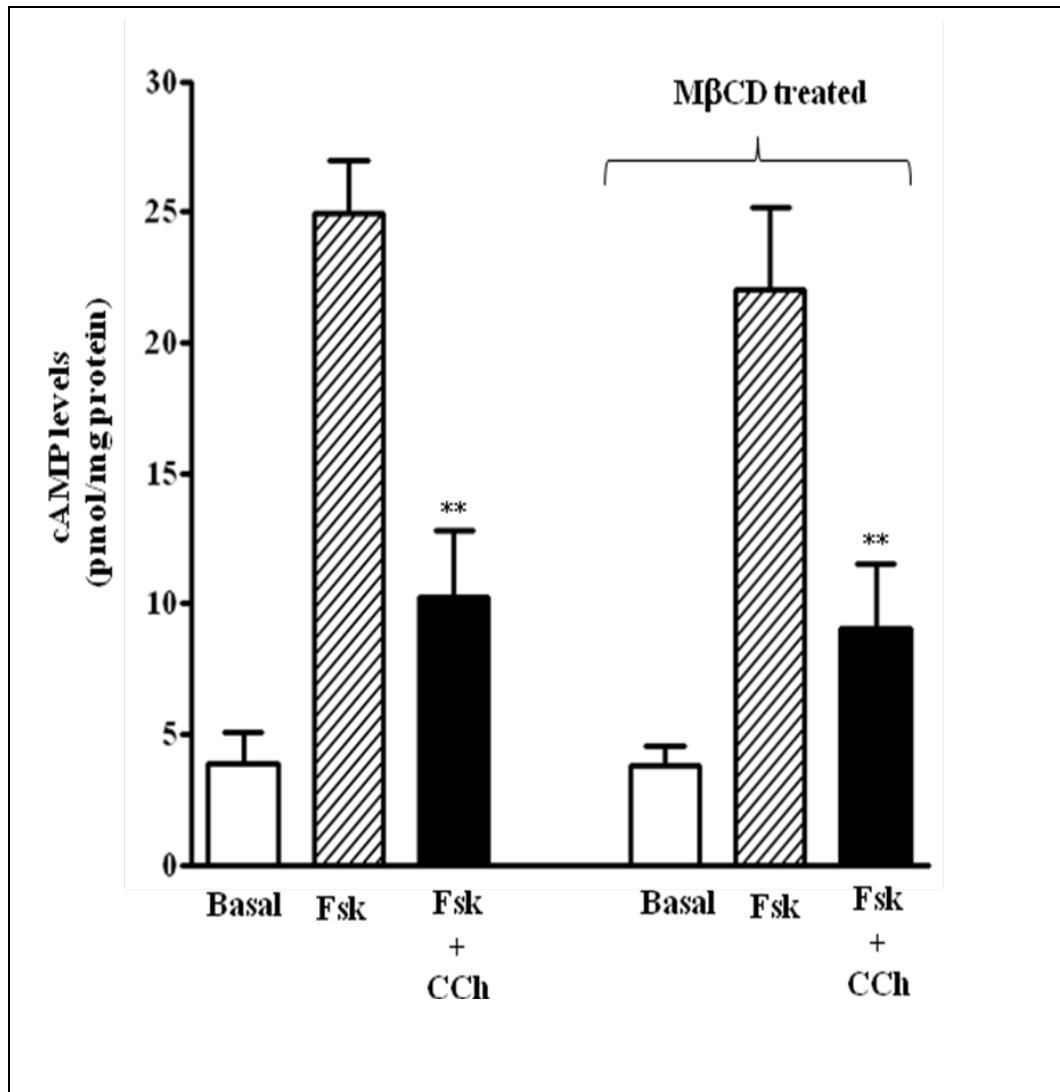


Fig. 24. Lack of effect on m2-mediated inhibition of adenylyl cyclase activity by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. After plasmid transfection, cells were treated with forskolin (10 μ M) for 10 min in the presence or absence of CCh (0.1 μ M) and adenylyl cyclase activity was measured as increase in cAMP formation by radioimmunoassay. Forskolin caused an increase in cAMP formation and the increase was similar in both groups. Carbachol caused a significant inhibition of forskolin-stimulated cAMP formation and the extent of inhibition was similar in control cells and in cells transfected with caveolin-1 siRNA. Results are expressed as pmol/mg protein. Values are means \pm S.E of 4 experiments. ** Significant inhibition of forskolin-stimulated cAMP by CCh in both control and caveolin-1 siRNA transfected cells ($P < 0.001$).

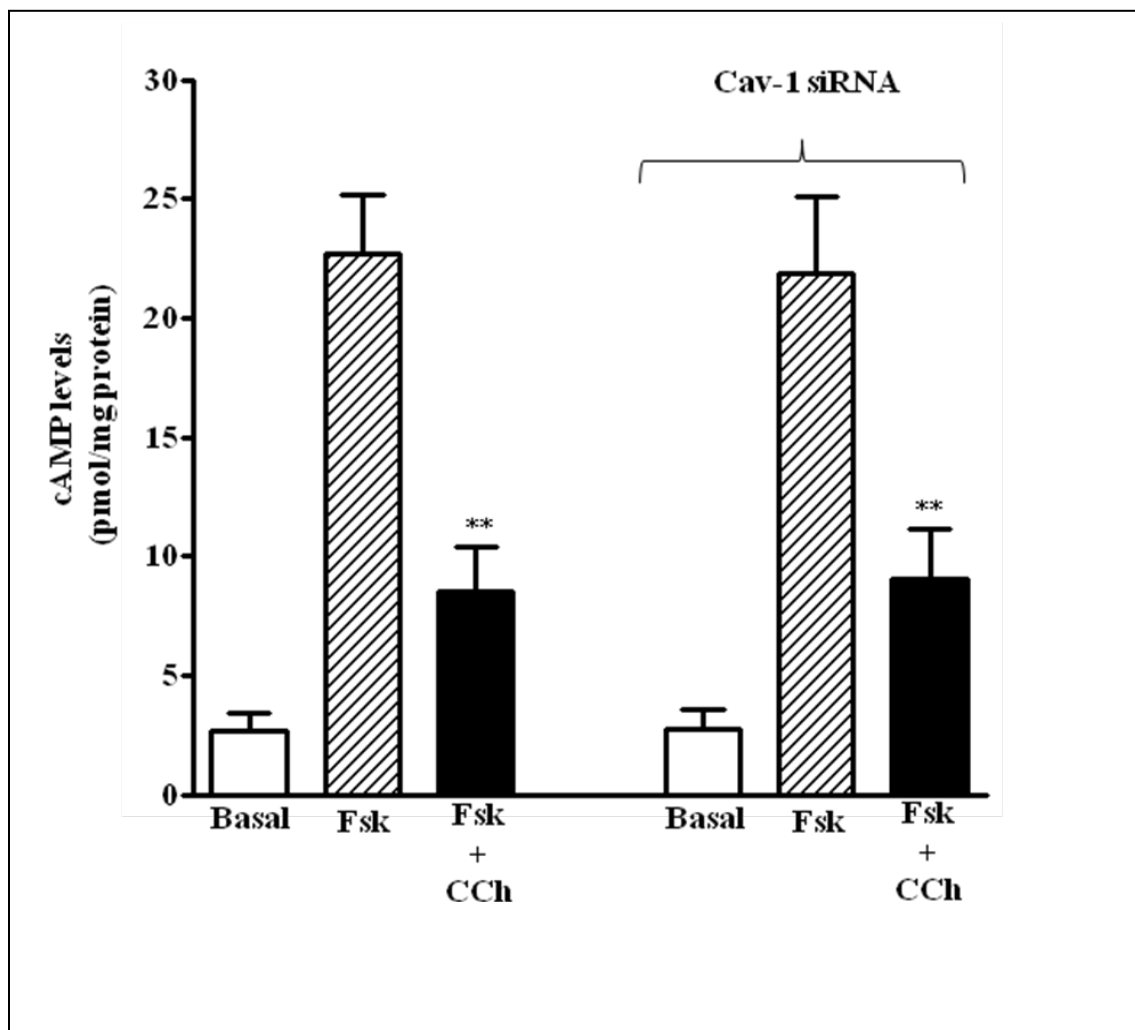
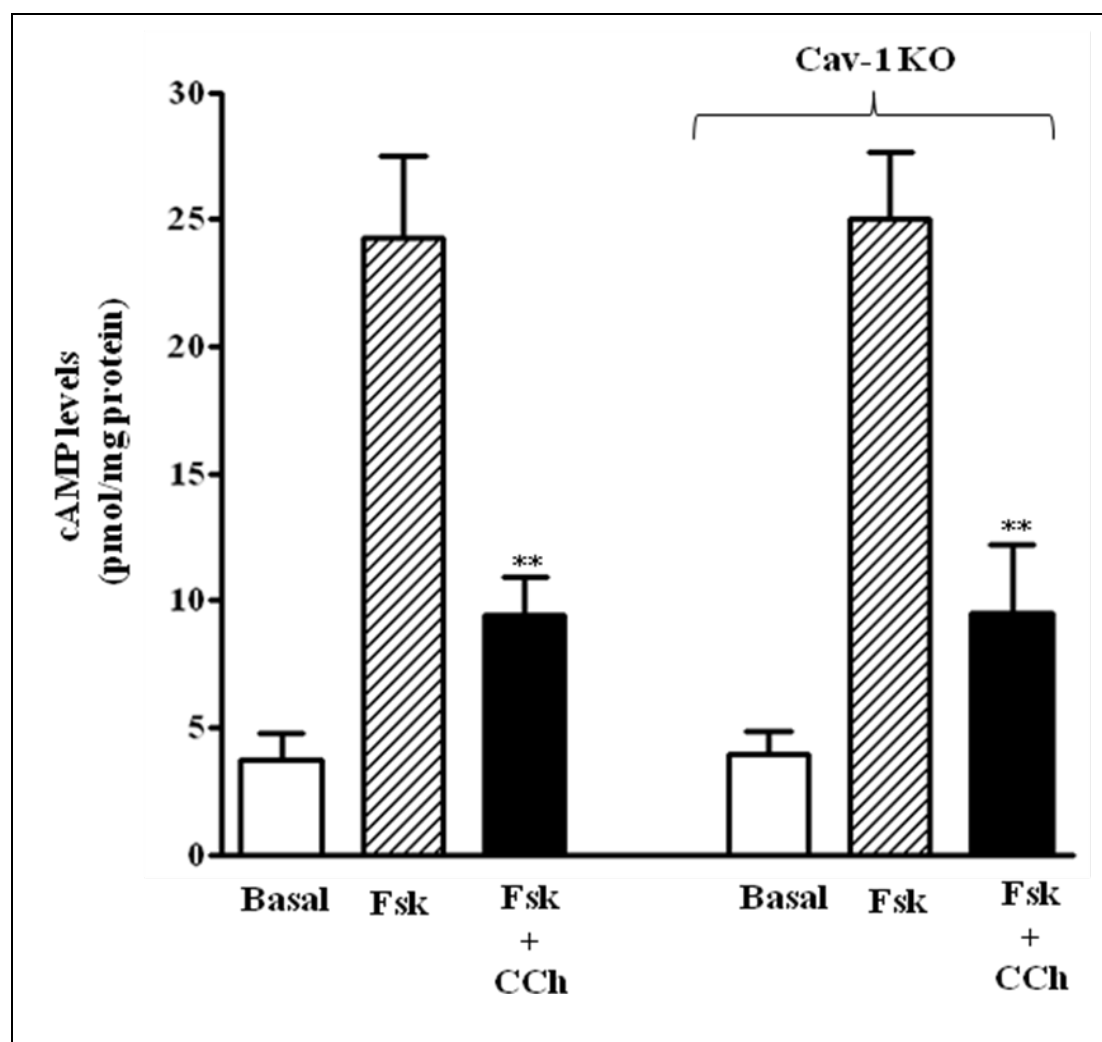


Fig. 25. Lack of effect on m2-mediated inhibition of adenylyl cyclase (AC) activity by caveolin-1 KO.

Gastric muscle isolated from wild type and caveolin-1 KO mice were treated with forskolin (10 μ M) for 10 min in the presence or absence of CCh (0.1 μ M) and adenylyl cyclase activity was measured as increase in cAMP formation by radioimmunoassay. Forskolin caused an increase in cAMP formation and the increase was similar in both groups. Carbachol caused a significant inhibition of forskolin-stimulated cAMP formation and the extent of inhibition was similar in wild type and caveolin-1 KO mice. Results are expressed as pmol/mg protein. Values are means \pm S.E of 4 experiments. ** Significant inhibition of forskolin-stimulated cAMP formation by CCh in muscle cells from both wild type and caveolin-1 KO mice ($P < 0.001$).



III. 6. Regulation of muscarinic m2 and m3 receptor internalization by caveolin-1.

Differential regulation of m2 and m3 receptor signaling raised the possibility that caveolin-1 is selectively associated with m3 receptors, but not m2 receptors. To examine this notion, association of m3 and m2 receptors with caveolin-1 was examined by western blot analysis of muscle homogenates fractionated by sucrose density gradient centrifugation. As shown previously caveolin-1 was selectively present in low buoyant fractions 4-5. In the unstimulated state both m2 and m3 receptors are present in high-density gradient fractions 9-12 and absent from the caveolar fractions 4-5. However, treatment of cells with CCh for 10 min caused translocation of m2, but not m3 receptors to caveolar fractions (Fig 26).

Since internalization of several G protein-coupled receptors utilize both clathrin and clathrin-independent viz. caveolae-dependent pathways, we tested the hypothesis that m2 receptors are selectively internalized via caveolae-dependent pathway (Fig. 27). Internalization was measured as decrease in radioligand ($[^3H]$ Scopolamine) binding to surface receptors after pretreatment of cells with CCh (0.1 μ M) for 20 min. Previous studies have shown that 20-min treatment with ligand causes maximum internalization of G protein coupled receptors such as VAPC₂, and CB₁ receptors (Mahavadi S 2004; Murthy, Mahavadi et al. 2008). The role of caveolae/caveolin-1 in the regulation of internalization was examined using M β CD in freshly dispersed muscle cells and caveolin-1 siRNA in cultured muscle cells. Limited yield of muscle cells from control and caveolin-1 KO mice precluded the use of this approach for binding studies. An essential step in the caveolae-dependent internalization process involves activation of Src kinase and phosphorylation of caveolar coat proteins at Tyr¹⁴ (Fagerholm, Ortegren et al. 2009). A selective Src kinase inhibitor PP2 was used as an additional tool to examine the caveolae-dependent pathway.

Specific binding was calculated as the difference between binding in the absence and in the presence (non-specific binding) of (10 μ M) CCh. Binding to m2 receptors was examined in the presence of m3 receptor antagonist, 4-DAMP (0.1 μ M), whereas binding to m3 receptor was examined in the presence of m2 receptor antagonist methoctramine (0.1 μ M). Binding to m2 receptors or m3 receptors was expressed as percent of both total (m2 and m3 receptor) specific binding.

III. 6a. Inhibition of m2 receptor internalization by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with M β CD (10 mM) each for 30 min. Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min and after that the cells are washed and used for radioligand binding studies.

Binding to m2 receptors was $68 \pm 5\%$ of total specific binding ($1,782 \pm 154$ cpm/mg protein) in control cells and $66 \pm 5\%$ of total specific binding ($1,628 \pm 186$ cpm/ mg protein) in cells treated with M β CD. These results suggest that either total binding or binding to m2 receptors was not affected by treatment of cells with M β CD. To examine m2 receptor internalization, muscle cells were treated with CCh (0.1 μ M) for 20 min, washed free of ligand, and then binding was measured in the presence of m3 receptor antagonist 4-DAMP (0.1 μ M). Treatment of control cells with CCh attenuated binding to m2 receptors to $21 \pm 4\%$ of total binding ($69 \pm 5\%$ attenuation compared to control m2 receptor binding) reflecting internalization of m2 receptors. In contract, when cells are pretreated with M β CD, CCh treatment had no significant effect on binding to m2 receptor ($62 \pm 7\%$ of total specific binding), which is similar to

binding to m2 receptors in the absence of CCh treatment ($66\pm 5\%$ of total specific binding) (Fig. 28). These results suggest that m2 receptor internalization was mediated via caveolar-dependent pathway in gastric smooth muscle.

III. 6b. Inhibition of m2 receptor internalization by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Suppression of caveolin-1 expression was verified by western blot analysis and immunofluorescence. Each group was further divided into two sets and one set in each group was incubated with CCh ($0.1\ \mu\text{M}$) for 20 min and after that the cells are washed and used for radioligand binding studies.

Binding to m2 receptors was $67\pm 6\%$ of total specific binding ($1,814\pm 205$ cpm/mg protein) in control cells and $66\pm 3\%$ of total specific binding ($1,758\pm 254$ cpm/ mg protein) in cells transfected with caveolin-1 siRNA. These results suggest that either total binding or binding to m2 receptors was not affected by transfection of cells with caveolin-1 siRNA. To examine m2 receptor internalization, muscle cells were treated with CCh ($0.1\ \mu\text{M}$) for 20 min, washed free of ligand, and then binding was measured in the presence of m3 receptor antagonist 4-DAMP ($0.1\ \mu\text{M}$). Treatment of control cells with CCh ($0.1\ \mu\text{M}$) attenuated binding to m2 receptors to $26\pm 5\%$ of total binding ($61\pm 6\%$ attenuation compared to control m2 receptor binding) reflecting internalization of m2 receptors. In contrast, cells transfected with caveolin-1 siRNA, CCh treatment had no significant effect on binding to m2 receptor ($64\pm 6\%$ of total specific binding), which is similar to binding to m2 receptors in the absence of CCh treatment

($67\pm 6\%$ of total specific binding) (Fig. 29). These results suggest that m2 receptor internalization was mediated via caveolin-1-dependent pathway in gastric smooth muscle.

III. 6c. Inhibition of m2 receptor internalization by Src kinase inhibitor, PP2.

To examine the involvement of Src kinase in m2 receptor internalization, a selective Src kinase inhibitor PP2 ($1\text{ }\mu\text{M}$) was used. Binding to m2 receptors was $67\pm 8\%$ of total specific binding ($1689\pm 225\text{ cpm/mg protein}$) in control cells and $64\pm 5\%$ of total specific binding ($1567\pm 196\text{ cpm/mg protein}$) in cells treated with PP2. These results suggest that either total binding or binding to m2 receptors was not affected by PP2. Treatment of control cells with CCh ($0.1\text{ }\mu\text{M}$) attenuated binding to m2 receptors to $25\pm 5\%$ of total binding ($63\pm 4\%$ attenuation compared to control m2 receptor binding) reflecting internalization of m2 receptors. In contrast, cells pretreated with PP2, CCh ($0.1\text{ }\mu\text{M}$) treatment had no significant effect on binding to m2 receptor ($59\pm 7\%$ of total specific binding), which is similar to binding to m2 receptors in the absence of CCh treatment ($67\pm 8\%$ of total specific binding) (Fig. 30). These results suggest that m2 receptor internalization was mediated via caveolin-1-dependent pathway involving Src kinase in gastric smooth muscle.

III. 6d. Lack of effect on m3 receptor internalization by methyl β -cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with M β CD (10 mM) each for 30 min. Each group was further divided into two sets and one set in each group was incubated with CCh ($0.1\text{ }\mu\text{M}$) for 20 min and after that the cells were washed and used for radioligand binding studies. Binding to m3 receptors was

examined in the presence of methoctramine (0.1 μ M) and expressed as percent of total specific binding.

Binding to m3 receptors was $30\pm4\%$ of total specific binding ($1,782\pm154$ cpm/mg protein) in control cells and $28\pm4\%$ of total specific binding ($1,628\pm186$ cpm/ mg protein) in cells treated with M β CD. These results suggest that binding to m3 receptors was not affected by treatment of cells with M β CD. Pretreatment of control cells with CCh (0.1 μ M) attenuated binding to m3 receptors to $10\pm2\%$ of total binding ($66\pm4\%$ attenuation compared to control m3 receptor binding) in control cells and to $11\pm2\%$ of total binding ($61\pm3\%$ attenuation) in cell treated with M β CD (Fig. 31). The decrease in binding to m3 receptor reflected internalization of m3 receptors and this was not affected by M β CD, suggesting that m3 receptor internalization was mediated via caveolae-independent pathway in gastric smooth muscle.

III. 6e. Lack of effect on m3 receptor internalization by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Suppression of caveolin-1 expression was verified by western blot analysis and immunofluorescence. Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min and after that the cells are washed and used for radioligand binding studies. Binding to m3 receptors was examined in the presence of methoctramine (0.1 μ M) and expressed as percent of total specific binding.

Binding to m3 receptors was $33\pm4\%$ of total specific binding ($1,814\pm205$ cpm/mg protein) in control cells and $31\pm2\%$ of total specific binding ($1,758\pm254$ cpm/ mg protein) in cells transfected with caveolin-1 siRNA. These results suggest that binding to m3 receptors were

not affected by transfection of cells with caveolin-1 siRNA. Pretreatment of control cells with CCh attenuated binding to m3 receptors to $10\pm3\%$ of total binding ($69\pm5\%$ attenuation compared to control m3 receptor binding) in control cells and to $11\pm3\%$ of total binding ($64\pm4\%$ attenuation) in cells transfected with caveolin-1 siRNA (Fig. 32). The decrease in binding to m3 receptor reflected internalization of m3 receptors and this was not affected by caveolin-1 siRNA, suggesting that m3 receptor internalization was mediated via caveolin-1-independent pathway in gastric muscle.

III. 6f. Lack of effect on m3 receptor internalization by Src inhibitor, PP2.

To examine the involvement of Src kinase in m3 receptor internalization, a selective Src kinase inhibitor PP2 ($1\text{ }\mu\text{M}$) was used. Binding to m3 receptors was $31\pm4\%$ of total specific binding ($1689\pm225\text{ cpm/mg protein}$) in control cells and $29\pm4\%$ of total specific binding ($1567\pm196\text{ cpm/mg protein}$) in cells treated with PP2. These results suggest that binding to m3 receptors were not affected by treatment of cells with PP2. Pretreatment of control cells with CCh ($0.1\text{ }\mu\text{M}$) attenuated binding to m3 receptors to $11\pm2\%$ of total binding ($64\pm5\%$ attenuation compared to control m3 receptor binding) in control cells and to $16\pm4\%$ of total binding ($45\pm5\%$ attenuation) in cells transfected with caveolin-1 siRNA (Fig. 33). The decrease in binding to m3 receptor reflected internalization of m3 receptors and this was minimally, but significantly, affected by PP2, suggesting that m3 receptor internalization was partly mediated via Src kinase-independent pathway in gastric smooth muscle.

In summary, pretreatment of muscle cells with CCh caused internalization of both m2 and m3 receptors. Internalization of m2, but not m3 receptors was blocked in dispersed cells

treated with M β CD or in cultured muscle cells transfected with caveolin-1 siRNA. These results suggest that caveolin-1 positively regulates m2, but not m3 receptor internalization in gastric muscle.

Fig. 26. Localization of muscarinic m2 and m3 receptors in caveolar and non-caveolar fractions.

Freshly dispersed muscle cells were used for caveolae isolation using sucrose density gradient centrifugation. After centrifugation, 1 ml fractions were collected from the top. The density of buoyancy increases with the increase in fraction number. Equal amount of protein were subjected to electrophoresis. Localization of caveolin-1 and m2 and m3 receptors in different fractions was examined by western blot analysis using specific antibody. Caveolin-1 is localized in low buoyant fraction 4-and 5. In the basal state both m2 and m3 receptors are present in high buoyant fraction 9-12 and upon treatment of cells with CCh for 15 min, m2 but not m3 receptors are translocated to caveolar fractions. Results shown are representative of 3 different experiments

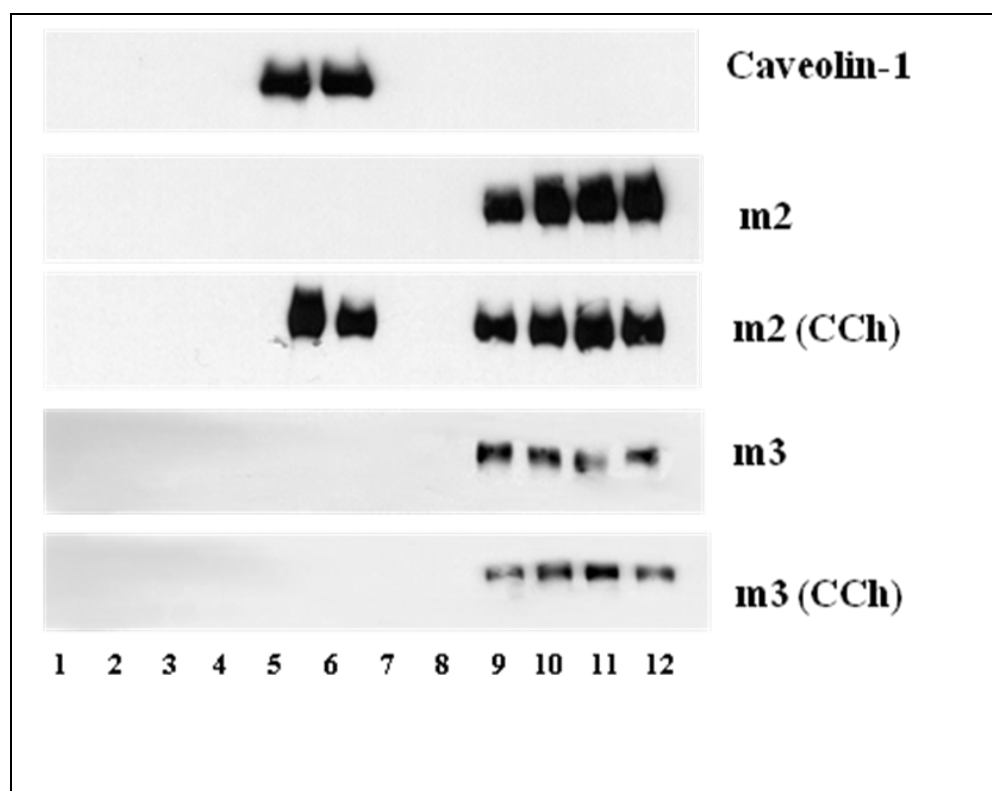
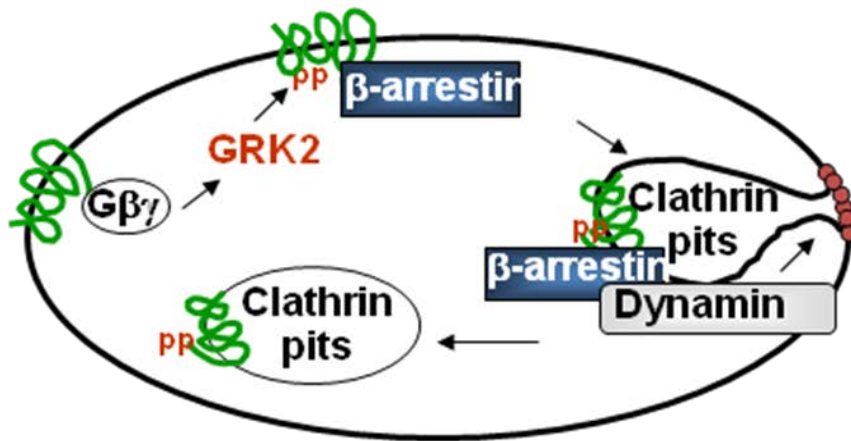


Fig. 27. Pathways for receptor internalization.

Internalization of G protein-coupled receptors is mediated by two major pathways: Clathrin-dependent pathway and clathrin-independent or caveolar-mediated pathway. Clathrin-dependent pathway involves phosphorylation of receptors by G protein coupled receptor kinases (GRKs) followed by binding of β -arrestin to the phosphorylated receptor, recruitment of clathrin to β -arrestin-bound receptors and formation of clathrin-coated pits and fission of clathrin pits with the help of dynamin. Caveolar-mediated pathway is independent of clathrins. Following receptor clustering in caveolar microdomains, Src mediated phosphorylation of caveolin-1 at Tyr¹⁴ leads to caveolae-detachment and internalization of receptors.

Clathrin-Dependent Pathway



Caveolin-Dependent Pathway

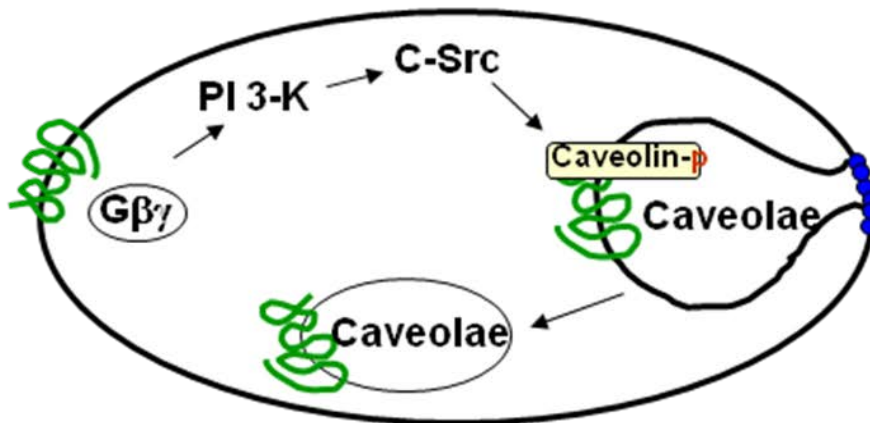


Fig. 28. Inhibition of m2 receptor internalization by methyl β cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with M β CD (10 mM) each for 30 min. Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min in the presence of 4-DAMP (0.1 μ M) to induce m2 receptor internalization. The cells are washed and incubated with [3 H]Scopolamine in the absence of 4-DAMP to measure binding to both m2 and m3 receptors (total) or in the presence of 4-DAMP to measure binding to m2 receptors. Specific binding was calculated as the difference between the binding in the absence of CCh and non-specific binding in the presence of 10 μ M CCh. Decrease in specific binding after CCh pretreatment represents receptor internalization. Results are expressed as percent of total (m2 and m3) specific binding. Values are means \pm S.E of three experiments. ** Significant decrease in binding compared to control binding in the absence of CCh pretreatment (P<0.001).

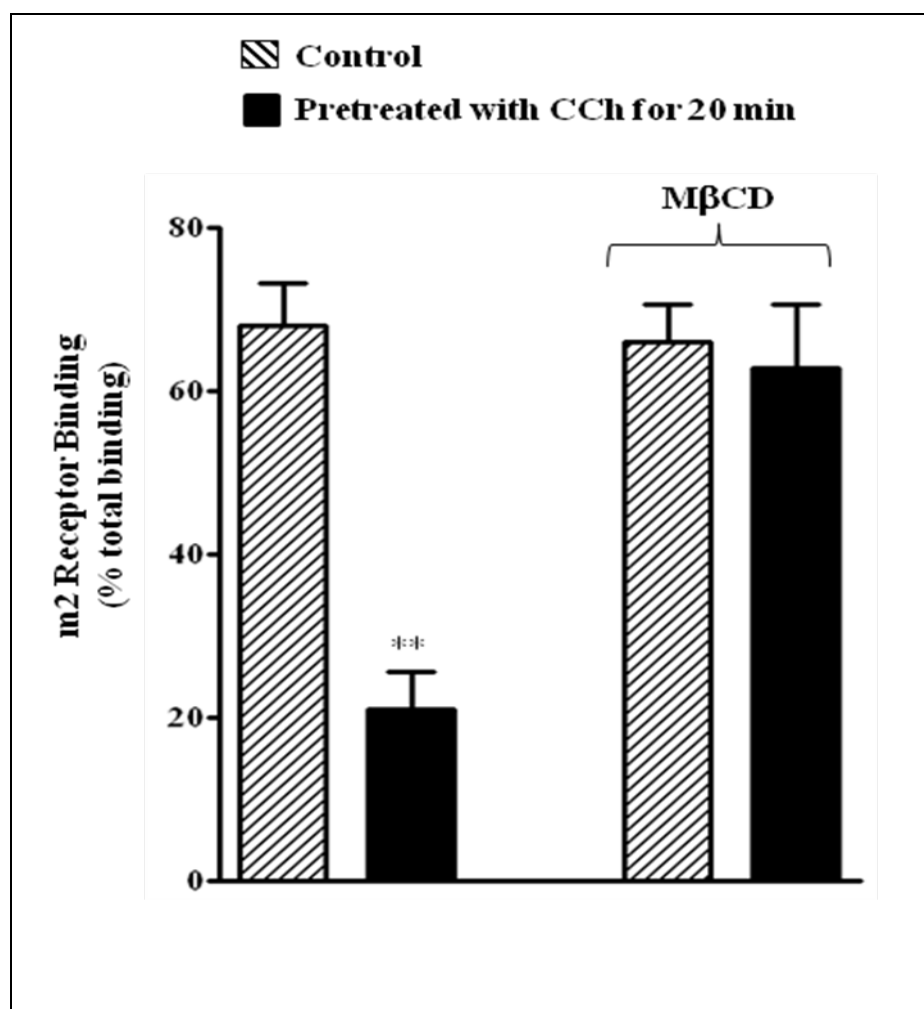


Fig. 29. Inhibition of m2 receptor internalization by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min in the presence of 4-DAMP (0.1 μ M) to induce m2 receptor internalisation. The cells are washed and incubated with [3 H]Scopolamine in the absence of 4-DAMP to measure binding to both m2 and m3 receptors (total) or in the presence of 4-DAMP to measure binding to m2 receptors. Specific binding was calculated as the difference between the binding in the absence of CCh and non-specific binding in the presence of 10 μ M CCh. Decrease in specific binding after CCh pretreatment represents receptor internalization. Results are expressed as percent of total (m2 and m3) specific binding. Values are means \pm S.E of three experiments. ** Significant decrease in binding compared to control binding in the absence of CCh pretreatment (P<0.001).

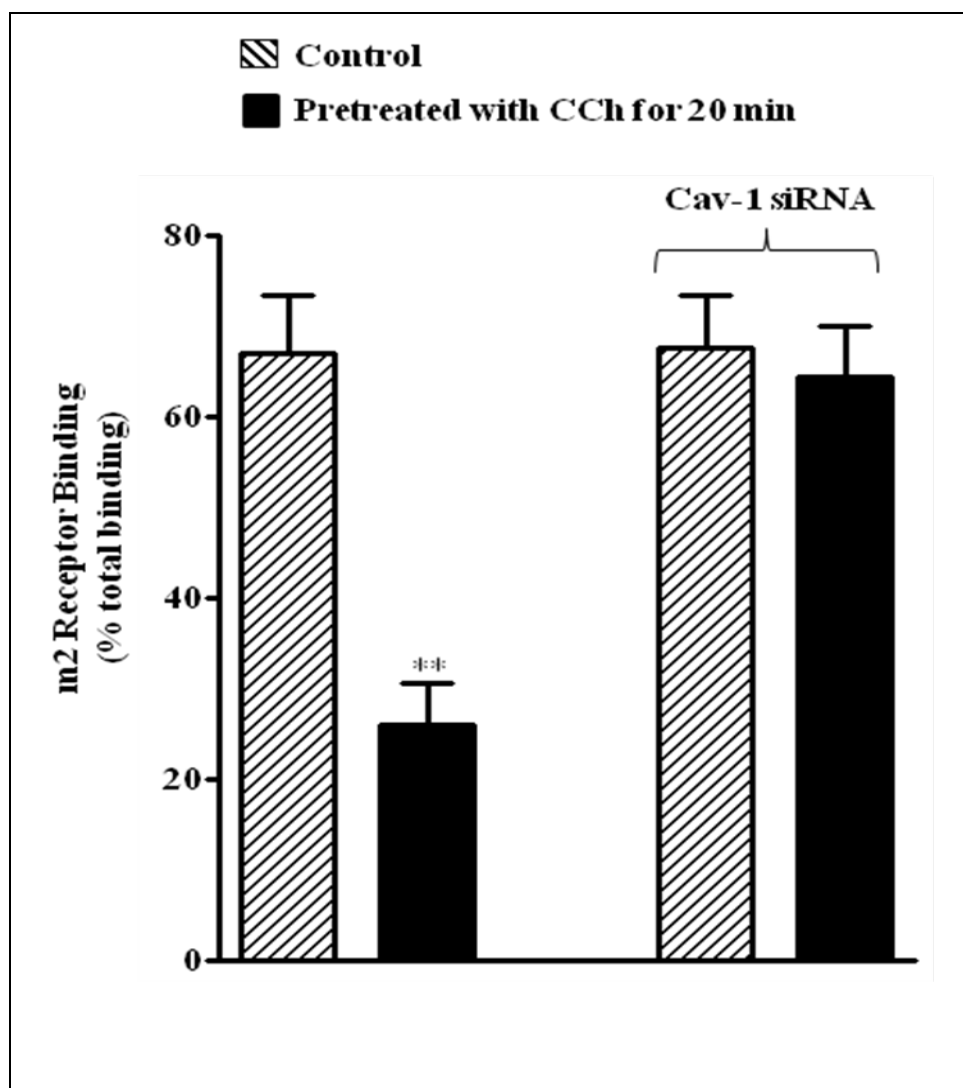


Fig. 30. Inhibition of m2 receptor internalization by Src kinase inhibitor, PP2.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with a selective Src kinase inhibitor PP2 (1 μ M). Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min in the presence of 4-DAMP (0.1 μ M) to induce m2 receptor internalisation. The cells are washed and incubated with [3 H]scopolamine in the absence of 4-DAMP to measure binding to both m2 and m3 receptors (total) or in the presence of 4-DAMP to measure binding to m2 receptors. Specific binding was calculated as the difference between the binding in the absence of CCh and non-specific binding in the presence of 10 μ M CCh. Decrease in specific binding after CCh pretreatment represents receptor internalization. Results are expressed as percent of total (m2 and m3) specific binding. Values are means \pm S.E of three experiments. ** Significant decrease in binding compared to control binding in the absence of CCh pretreatment ($P < 0.001$).

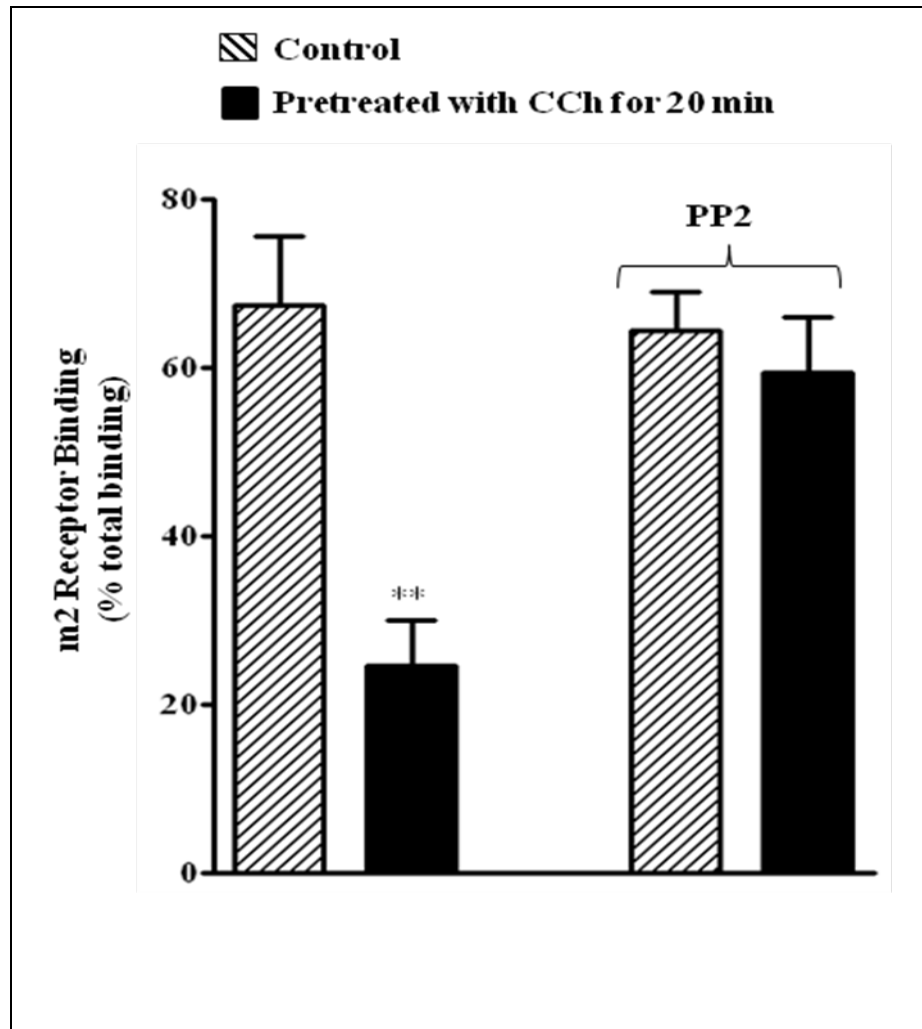


Fig. 31. Lack of effect on m3 receptor internalization by methyl β cyclodextrin.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with M β CD (10 mM) each for 30 min. Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min in the presence of methoctramine (0.1 μ M) to induce m3 receptor internalisation. The cells are washed and incubated with [3 H]Scopolamine in the absence of methoctramine to measure binding to both m2 and m3 receptors (total) or in the presence of methoctramine to measure binding to m3 receptors. Specific binding was calculated as the difference between the binding in the absence of CCh and non-specific binding in the presence of 10 μ M CCh. Decrease in specific binding after CCh pretreatment represents receptor internalization. Results are expressed as percent of total (m2 and m3) specific binding. Values are means \pm S.E of three experiments. ** Significant decrease in binding compared to control binding in the absence of CCh pretreatment ($P < 0.001$).

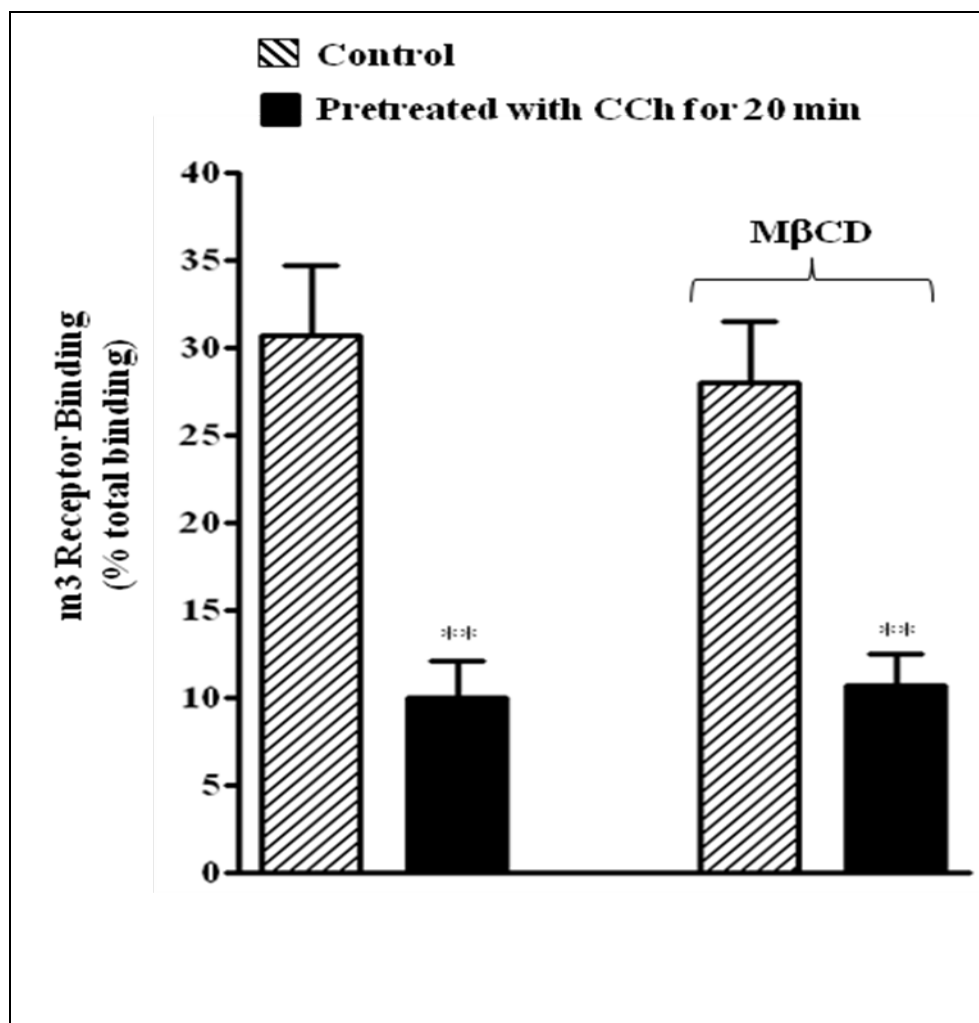


Fig. 32. Lack of effect on m3 receptor internalization by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min in the presence of methoctramine (0.1 μ M) to induce m3 receptor internalisation. The cells are washed and incubated with [3 H]*Scopolamine* in the absence of methoctramine to measure binding to both m2 and m3 receptors (total) or in the presence of methoctramine to measure binding to m3 receptors. Specific binding was calculated as the difference between the binding in the absence of CCh and non-specific binding in the presence of 10 μ M CCh. Decrease in specific binding after CCh pretreatment represents receptor internalization. Results are expressed as percent of total (m2 and m3) specific binding. Values are means \pm S.E of three experiments. ** Significant decrease in binding compared to control binding in the absence of CCh pretreatment ($P < 0.001$).

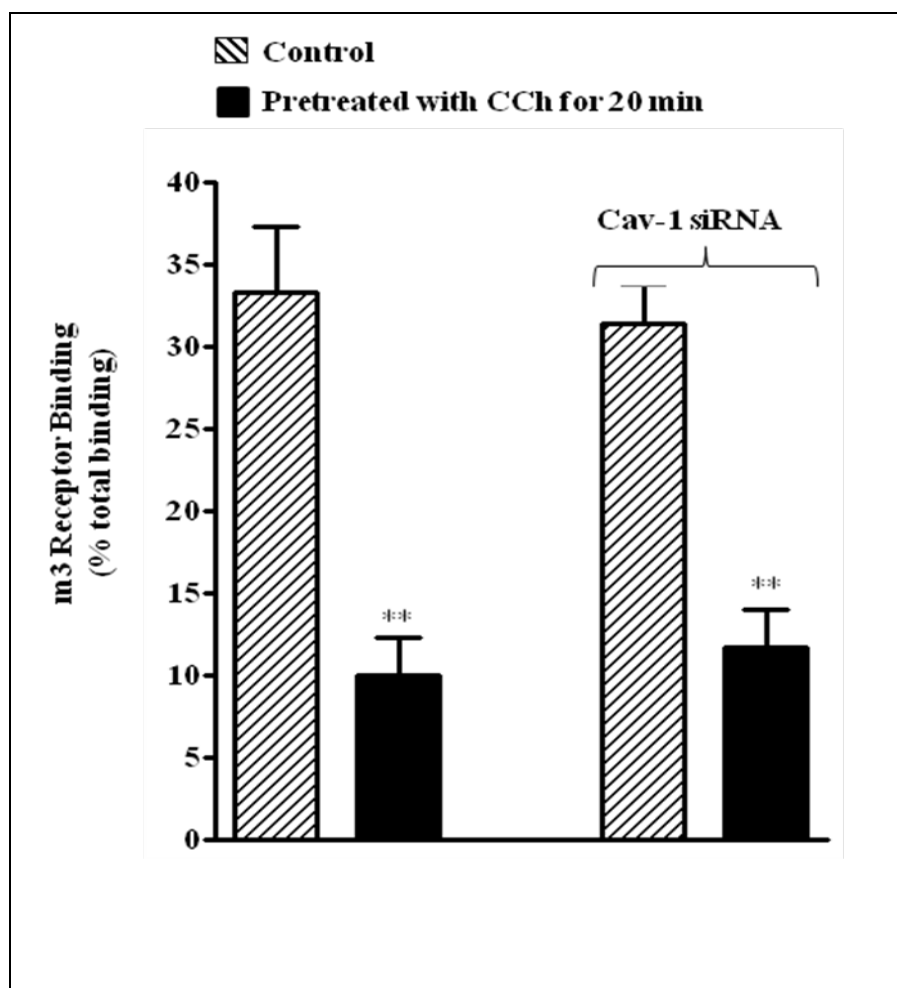
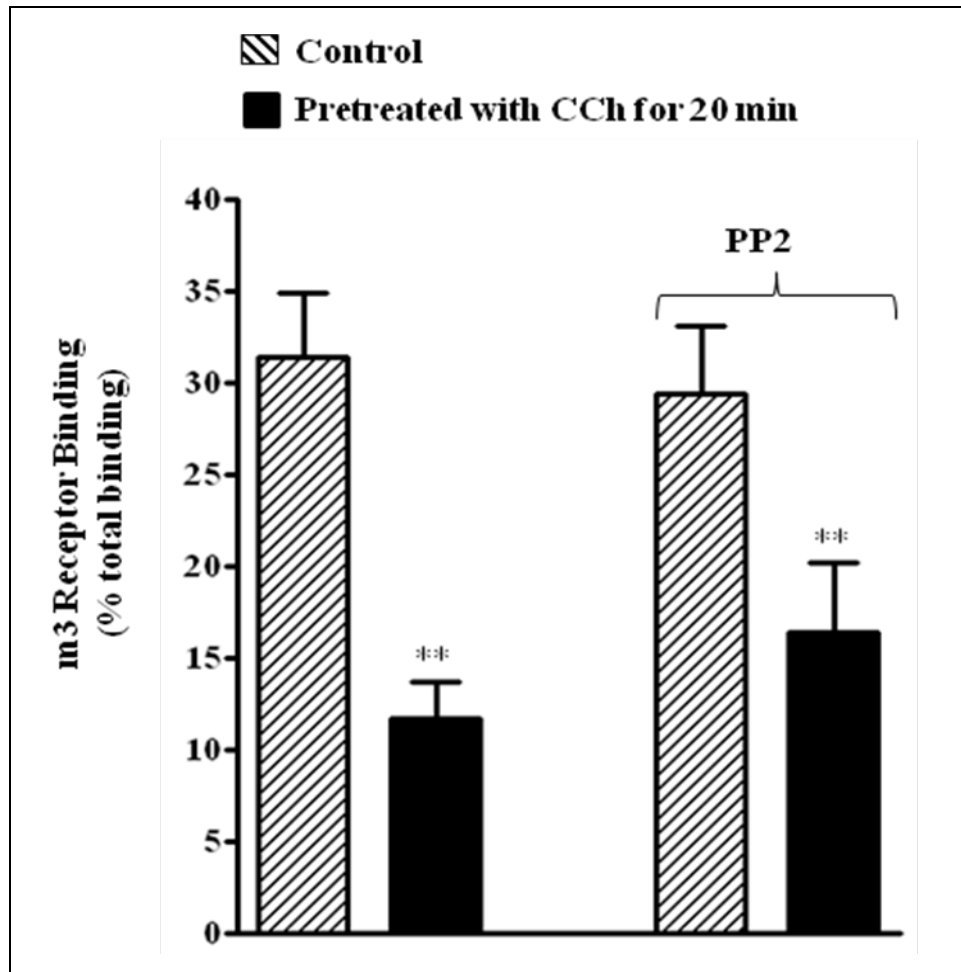


Fig. 33. Lack of effect on m3 receptor internalization by Src inhibitor, PP2.

Freshly dispersed gastric smooth muscle cells were divided into control and experimental group. The control group was incubated with DMSO and the experimental group was incubated with a selective Src kinase inhibitor PP2 (1 μ M). Each group was further divided into two sets and one set in each group was incubated with CCh (0.1 μ M) for 20 min in the presence of methoctramine (0.1 μ M) to induce m3 receptor internalisation. The cells are washed and incubated with [3 H]Scopolamine in the absence of methoctramine to measure binding to both m2 and m3 receptors (total) or in the presence of methoctramine to measure binding to m3 receptors. Specific binding was calculated as the difference between the binding in the absence of CCh and non-specific binding in the presence of 10 μ M CCh. Decrease in specific binding after CCh pretreatment represents receptor internalization. Results are expressed as percent of total (m2 and m3) specific binding. Values are means \pm S.E of three experiments. ** Significant decrease in binding compared to control binding in the absence of CCh pretreatment ($P < 0.001$).



III. 7. Regulation of excitation-transcription coupling by caveolin-1.

Modulation of smooth muscle phenotype occurs as results of modulation of signaling pathways that regulate smooth muscle contractile proteins such as smooth muscle γ -actin (SM- γ actin), SM-myosin heavy chain (SM-MHC), calponin, and caldesmon. Expression of contractile proteins is regulated by the key transcriptional factors such as serum response factor (SRF) and transcriptional co-activators such as myocardin (Pipes, Creemers et al. 2006). The Ca^{2+} and RhoA/Rho kinase pathways have been associated with the regulation of SRF/myocardin activity. Recent studies have revealed that the number of caveolae, and their marker protein caveolin-1 is greatly increased as smooth muscle cells acquire a contractile phenotype (Gosens, Stelmack et al. 2011) (Fig. 34). Since there is decrease in RhoA/Rho kinase pathway with caveolin-1 suppression, we tested the hypothesis that caveolin-1 positively regulates contractile protein expression in smooth muscle. Expression of SRF, myocardin, γ -actin and caldesmon was measured by real time RT-PCR and western blot in cultured muscle cells transfected with control siRNA or caveolin-1 siRNA. mRNA expression of contractile proteins such as γ -actin and caldesmon (Fig. 36) and the transcription factors SRF (% inhibition) and myocardin (% inhibition) (Fig. 35) are significantly inhibited in cells transfected with caveolin-1 siRNA compared to cells transfected with control siRNA. This inhibition of mRNA expression paralleled inhibition of protein expression of γ -actin, caldesmon, SRF and myocardin in cells transfected with caveolin-1 siRNA compared to cells transfected with control siRNA. These results suggest that a positive regulation of contractile proteins expression and the transcription factors that regulate contractile protein expression is exerted by caveolin-1 in gastric smooth muscle. The decrease in the expression of contractile proteins by caveolin-1 suppression is accompanied by an increase in proliferative signals such as epidermal growth factor receptor

phosphorylation and ERK1/2 activity. Basal level of EGF receptor phosphorylation was significantly higher ($65\pm 13\%$ increase) in cells transfected with caveolin-1 siRNA. Treatment with EGF caused a significant increase ($138\pm 18\%$ increase above basal levels, $p<0.01$) in EGF receptor phosphorylation in control cells. The effect of EGF was significantly augmented in cells transfected with caveolin-1 siRNA ($218\pm 21\%$ increase above basal, $58\pm 6\%$ augmentation compared to EGF response in control cells) (Fig. 37). Consistent with increase in EGF receptor phosphorylation, basal ERK1/2 activity was significantly higher in cells transfected with caveolin-1 siRNA (control cells 1430 ± 138 cpm/mg protein; 2336 ± 351 cpm/mg protein; $62\pm 4\%$ increase, $p<0.01$). Treatment with EGF caused a significant increase ($151\pm 13\%$ increase above basal levels, $p<0.01$) in ERK1/2 activity in control cells. The effect of EGF was significantly augmented in cells transfected with caveolin-1 siRNA (235 ± 21 increase above basal, $57\pm 4\%$ augmentation compared to EGF response in control cells) (Fig. 38).

In summary, expression of contractile proteins, γ -actin and caldesmon and the transcription factors SRF and myocardin that regulate the expression of contractile proteins are down regulated in cells transfected with caveolin-1 siRNA. This is accompanied by an increase in basal and EGF-stimulated EGF receptor phosphorylation and ERK1/2 activity in cells transfected with caveolin-1 siRNA. These results suggest that caveolin-1 negatively regulates proliferative phenotype and positively regulates contractile phenotype in gastric muscle.

Fig. 34. Excitation-contraction coupling in smooth muscle.

Expression of smooth muscle specific genes is essential for maintaining its contractile phenotype. Ca^{2+} and RhoA-dependent signaling pathways positively regulate the expression of smooth muscle specific genes through transcription factors such as serum response factor (SRF) and myocardin that selectively binds to CArG elements on promoter region. In contrast, signaling through growth factors promotes smooth muscle proliferation by suppressing SRF and myocardin activity.

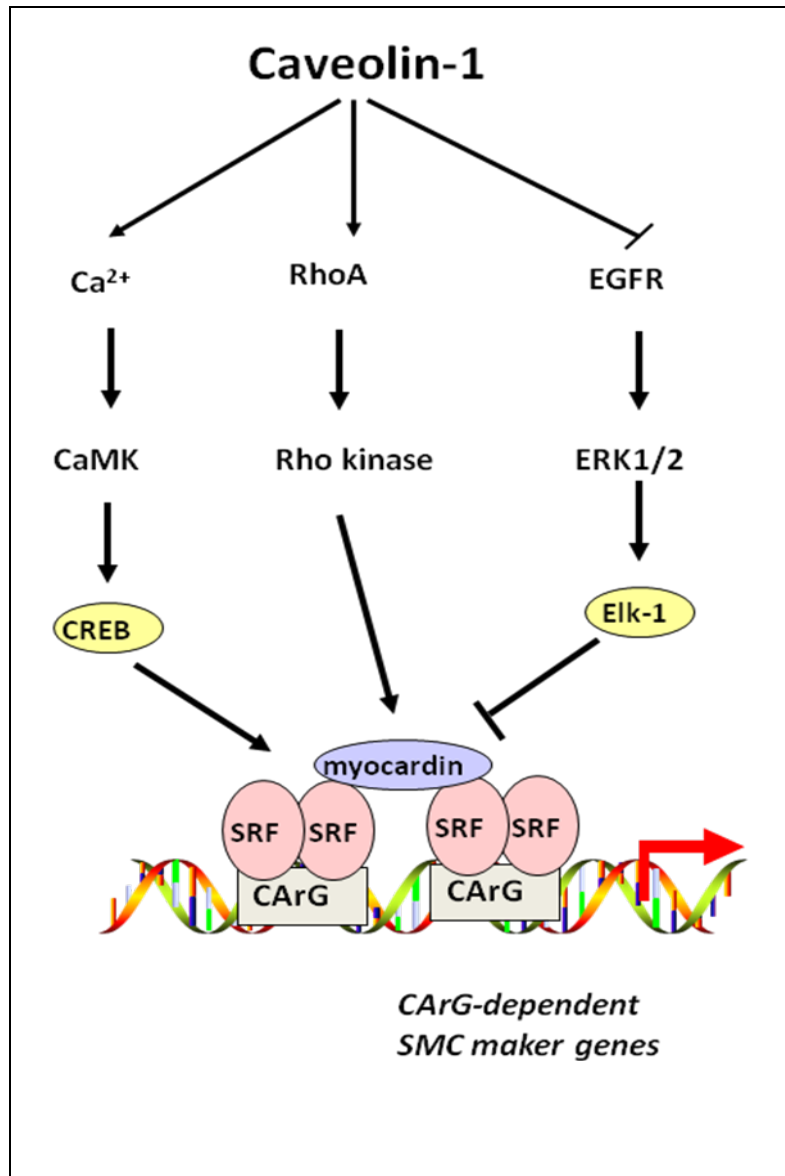


Fig. 35. Inhibition of serum response factor (SRF) and myocardin expression by caveolin-1 siRNA.

Total RNA was isolated from smooth muscle cells transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA transfected cells using RNAqueous prep kits (Ambion, Austin, Tx) and reverse transcribed using 2 µg of total RNA using qScript cDNA prep kits (Quanta, Gaithersburg, MD). The cDNA was amplified with specific primers for serum responsive factor (SRF) and myocardin. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure RNA levels of SRF and myocardin. For each cDNA sample, real-time PCR was conducted in a 20-µl-reaction volume containing QuantitectTM SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in gene expression in caveolin-1 siRNA transfected cells relative to that in control cells transfected with the vector alone. Values represent the means \pm SE of 3 separate experiments. **Inset:** Culture muscle cell lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of SRF and myocardin was analyzed using selective antibody. Results are expressed as fold differences in SRF or myocardin protein expression in caveolin-1 siRNA transfected cells relative to that in vector-transfected cells. Values represent the means \pm SE of 3 separate experiments. *Significant inhibition of expression in caveolin-1 siRNA transfected cells compared to expression in control cells ($p < 0.05$).

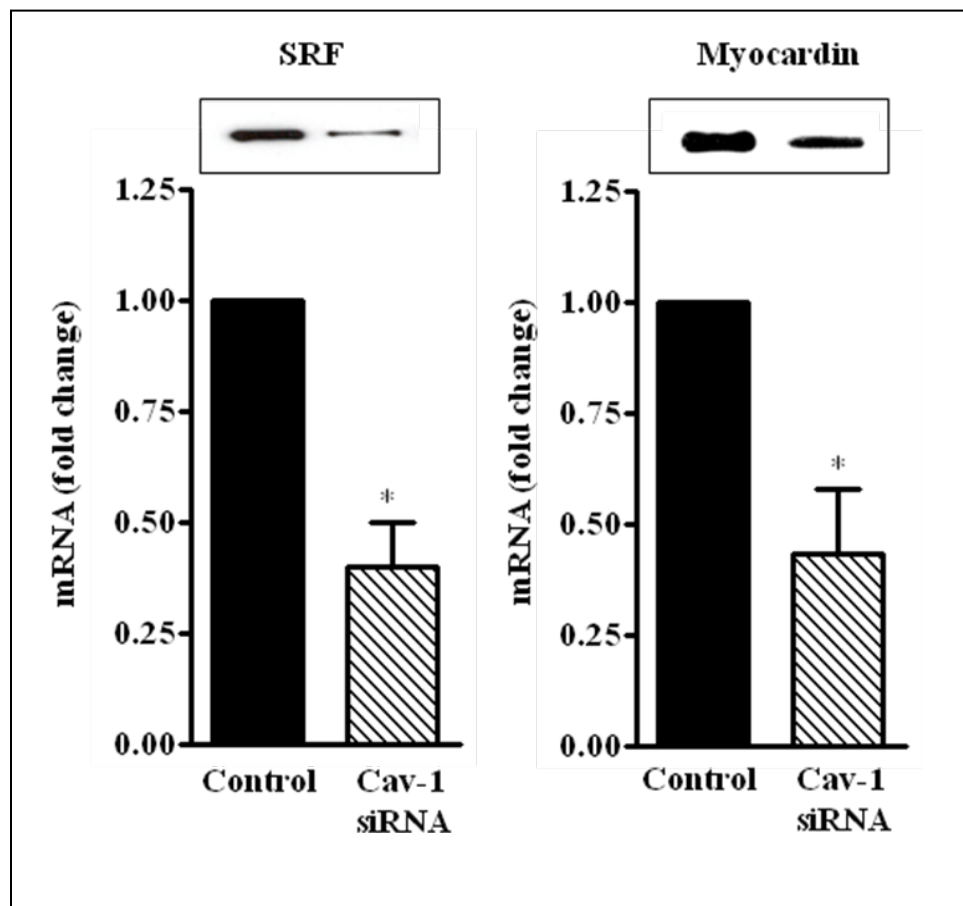


Fig. 36. Inhibition of γ -actin and caldesmon expression by caveolin-1 siRNA.

Total RNA was isolated from smooth muscle cells transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA transfected cells using RNAqueous prep kits (Ambion, Austin, Tx) and reverse transcribed using 2 μ g of total RNA using qScript cDNA prep kits (Quanta, Gaithersburg, MD). The cDNA was amplified with specific primers for γ -actin and caldesmon. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to measure RNA levels of SRF and myocardin. For each cDNA sample, real-time PCR was conducted in a 20- μ l-reaction volume containing QuantitectTM SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). Real-time PCR reactions were performed in triplicate. Results are expressed as fold differences in gene expression in caveolin-1 siRNA transfected cells relative to that in control cells transfected with the vector alone. Values represent the means \pm S.E of 3 separate experiments. **Inset:** Culture muscle cell lysates containing equal amounts of total proteins were separated with SDS-PAGE and expression of γ -actin and caldesmon was analyzed using selective antibody. Results are expressed as fold differences in SRF or myocardin protein expression in caveolin-1 siRNA transfected cells relative to that in control vector-transfected cells. Values represent the means \pm S.E of 3 separate experiments. * Significant inhibition of expression in caveolin-1 siRNA transfected cells compared to expression in control cells ($p < 0.05$).

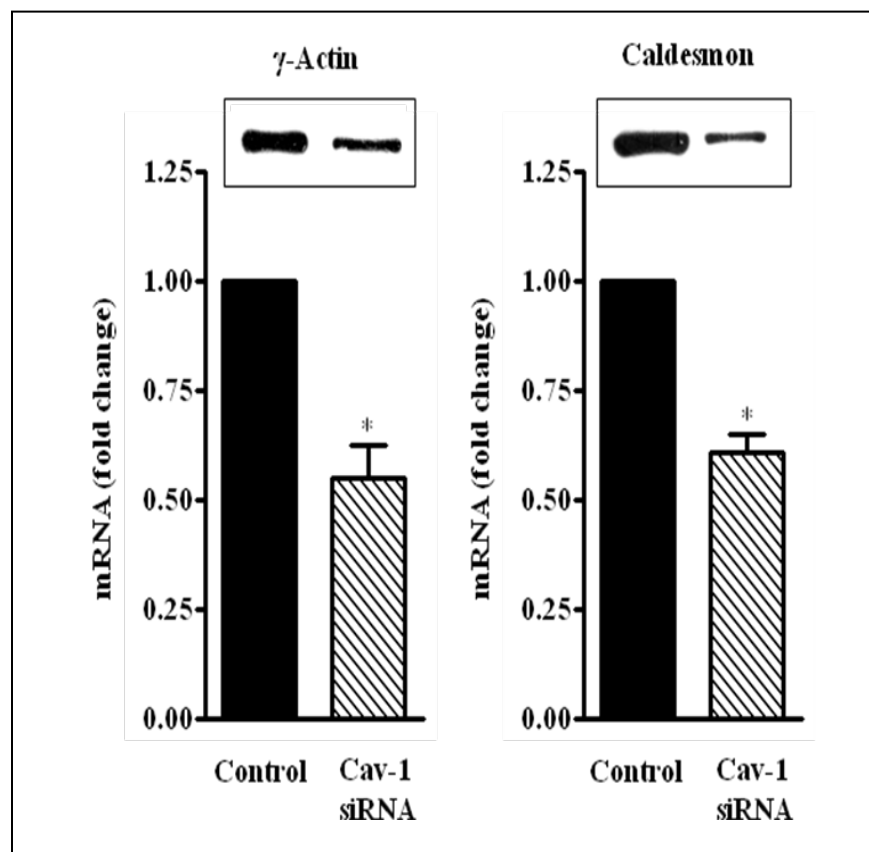


Fig. 37. Augmentation of EGF receptor phosphorylation by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Following transfection cells were treated with EGF (1 ng/ml) for 5 min. Cell lysates containing equal amounts of total proteins were separated on SDS-PAGE and the phosphorylation of EGF receptor was analysed using phospho-specific (Tyr¹⁰⁶⁸) antibody. Blots shown are representative of 3 different experiments. The bar graphs show quantification of the EFG receptor phosphorylation by densitometry. Values represent the means \pm S.E of 3 separate experiments. * Significant increase in EGF receptor phosphorylation compared to basal phosphorylation ($p < 0.01$). ** Significant increase in EGF receptor phosphorylation in response to EGF in cells transfected with caveolin-1 siRNA compared to response in control cells transfected with the vector alone ($p < 0.05$).

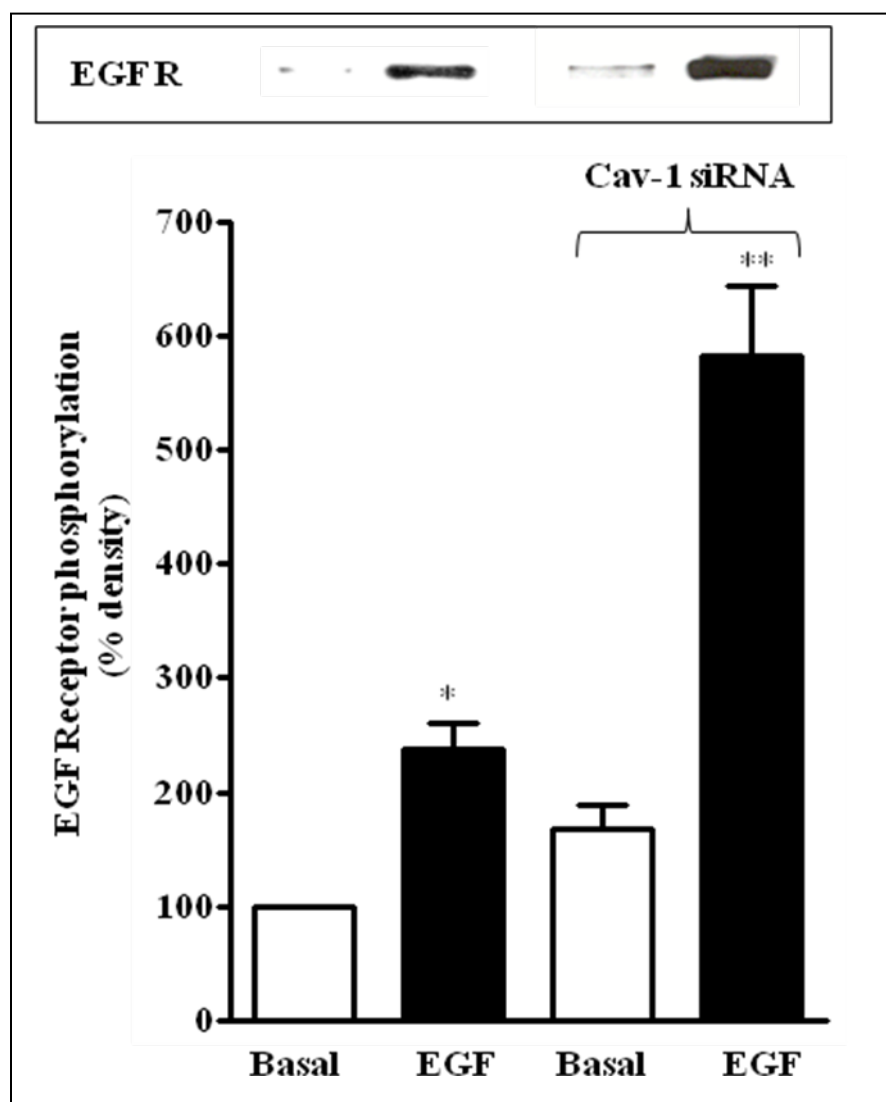
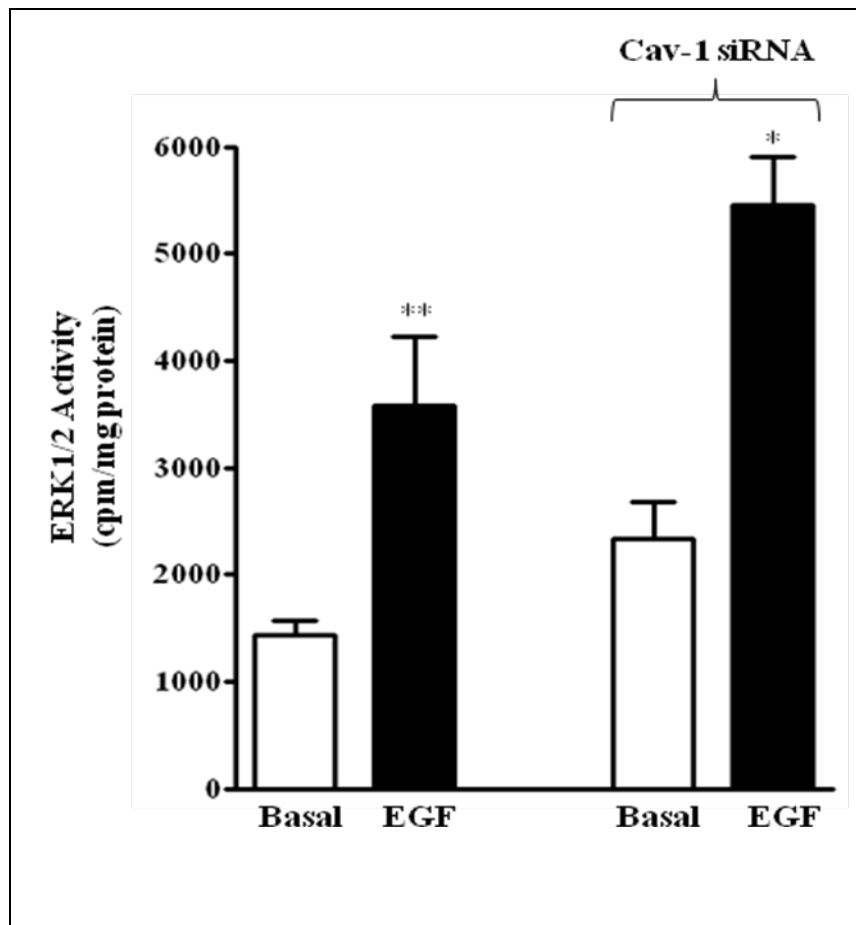


Fig. 38. Augmentation of ERK1/2 activity by caveolin-1 siRNA.

Primary culture of gastric smooth muscle cells were transfected with control pSIREN-DNR-DsRed vector and pSIREN-DNR-DsRed vector containing caveolin-1 specific siRNA for 48 h. Following transfection cells were treated with EGF (1ng/ml) for 5 min and ERK1/2 activity was measured using [γ -³²P]ATP by immunokinase assay. Results are expressed as cpm/mg protein. Values represent the means \pm S.E of 3 separate experiments. ** Significant increase in ERK1/2 activity compared to basal activity ($p < 0.01$). * Significant increase in ERK1/2 activity in response to EGF in cells transfected with caveolin-1 siRNA compared to response to EGF in control cells ($p < 0.05$).



CHAPTER IV: DISCUSSION

IV. DISCUSSION

The fundamental phenomenon of contraction in smooth muscle cells of the gastrointestinal tract is critical for propelling the intraluminal content from the oral to the caudal direction. Contraction is initiated by the release of contractile transmitters that mainly constitutes acetylcholine and tachykinins that are derived from enteric excitatory motor neurons. The signaling pathways activated by the contractile agonists also play an important role in the regulation of smooth muscle contractile phenotype by transcriptional and translational regulation of contractile protein expression. Both excitation-contraction and excitation-transcription coupling are, in turn, regulated by cell-extrinsic (extracellular matrix) and cell-intrinsic proteins.

Caveolae are integral parts of the smooth muscle membrane and several studies have demonstrated that caveolae act as signaling platforms or scaffolds that regulate cellular signaling through direct and indirect interaction with signaling proteins. All three main components of G protein coupled receptor system (receptor, G proteins and effector enzymes) are localized at the plasma membrane and their co-localization with caveolae is well suited for efficient transduction of signaling events. Caveolae contain the protein caveolins, and all three gene products of the caveolin proteins (caveolin-1, caveolin-2 and caveolin-3) are expressed in smooth muscle (Taggart 2001). Previous studies in both visceral and airway smooth muscle have shown that the relative mRNA expression levels were 15:1:1 for caveolin-1, caveolin-2 and caveolin-3, respectively (Shakirova, Bonnevier et al. 2006). Caveolin-1 is the predominant isoform in the smooth muscle and drives the formation of caveolae. However, little is known about the role of caveolin-1 in the regulation of excitation-contraction and excitation-transcription coupling in gastrointestinal smooth muscle. In the present study we initially characterized m2 and m3

receptor signaling in gastric smooth muscle and then examined the role of caveolin-1 in the regulation of signaling pathways mediated by muscarinic receptors. Since selective receptor-specific ligands are not available, m2 receptor-selective antagonist, methoctramine and m3 receptor-selective antagonist 4-DAMP were used to analyze the receptor-specific signaling. Previous studies in intestinal muscle have demonstrated that m3 are coupled to stimulation of PLC- β (PI hydrolysis) via G_q and RhoA-dependent activation of Rho kinase/ZIP kinase via G_{13} , whereas m2 receptors are coupled to inhibition of adenylyl cyclase via G_i (Murthy, Zhou et al. 2003; Uchiyama and Chess-Williams 2004). Similarly, m3-mediated stimulation of PI hydrolysis, and Rho kinase and ZIP kinase activity, and m2-mediated inhibition of AC activity was obtained in gastric muscle cells. Hence, these enzyme activities were used as readouts of $G_{q/13}$ -coupled m3 receptor activity and G_i -coupled m2 receptor activity to examine the role of caveolae/caveolin-1 on receptor-effectors coupling. Increase in MLC_{20} phosphorylation, a biochemical correlate, and decrease in muscle cell length were used as readouts of muscle function. Expression of key transcription factors such as SRF and myocardin and contractile proteins such as γ -actin, and caldesmon, and proliferative signals such as phosphorylation of EGF receptor and stimulation of ERK1/2 activity were measured as readouts to examine the role of caveolin-1 in the regulation of contractile phenotype. The role of caveolae/caveolin-1 was examined using three complimentary approaches: a) methyl β -cyclodextrin (M β CD) to deplete cholesterol in freshly dispersed muscle cells, b) caveolin-1 siRNA to suppress caveolin-1 expression in cultured muscle cells, and c) caveolin-1 knockout (KO) mice.

IV. 1. Signaling transduction by muscarinic receptors

Five distinct muscarinic receptors (m1-m5), derived from distinct gene products, have been characterized in mammalian tissues. Our initial studies characterized the expression of muscarinic receptors and the receptor-specific signaling pathways activated by CCh in rabbit gastric smooth muscle cells. Gastric smooth muscle expresses selectively m2 and m3 receptors and this was confirmed by RT-PCR, western blot and radioligand binding studies. Expression of m2 receptors is much greater (~70%) than m3 receptors (~30%). Although vascular, visceral and airway smooth muscle cells express mostly m2 and m3 receptors, the relative expression of m2 and m3 receptors varies depending on the tissue. In general, the expression of m2 receptors is more abundant than m3 receptors (Eglen, Reddy et al. 1994; Niihashi, Esumi et al. 2000; Wang, Han et al. 2001; Murthy, Zhou et al. 2003). Although the expression of m2 receptors is abundant than m3 receptors, contraction is exclusively mediated by m3 receptors in gastric muscle cells (Kitazawa, Hirama et al. 2008). Similar selective involvement of m3 receptors in contraction was demonstrated in vascular and other visceral smooth muscle cells. The contribution of m2 receptors to mediate muscle contraction varies depending on the smooth muscle tissue. Using receptor selective antagonists and transgenic mice, it was demonstrated that more than 90% of the contraction in response to CCh was mediated via m3 receptors in intestinal and bladder muscle, whereas only 50% contraction was mediated via m3 receptors in airway muscle and the remaining contraction was mediated via m2 receptors (Boulanger, Morrison et al. 1994; Chess-Williams, Chapple et al. 2001; Stengel, Yamada et al. 2002).

In the present study, we have demonstrated that CCh-stimulated PI hydrolysis and Rho Kinase and ZIP kinase activity were blocked by the selective m3 receptor antagonist, 4-DAMP, whereas CCh-induced inhibition of AC activity was blocked by the selective m2 receptor

antagonist, methoctramine and pertussis toxin. These results are consistent with the muscarinic receptor signaling in other visceral and vascular smooth muscle (Singer, Vang et al. 2002; Gerthoffer 2005; Unno, Matsuyama et al. 2006). Receptor-specific coupling to G proteins and activation of signaling pathways are not unique to muscarinic m2 and m3 receptors. Previous studies in smooth muscle have shown that similar receptor-specific coupling to distinct G proteins and signaling with 5-HT (5-HT₂ and 5-HT₄) receptors, adenosine (A₂ and A₁) receptors, shingosine-1-phosphate (S1P₁ and S1P₂) receptors, neuropeptide (NPY₁ and NPY₂) receptors, and endothelin (ET_A and ET_B) receptors (Kuemmerle, Murthy et al. 1995; Murthy and Makhoulf 1995; Murthy and Makhoulf 1998; Hersch, Huang et al. 2004; Misra, Murthy et al. 2004; Zhou and Murthy 2004).

Excitatory neurotransmitters such as acetylcholine and tachykinins mobilize intracellular Ca²⁺ via G_{α_q}-dependent stimulation of PLC-β1. Activation of PLC-β1 hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP₂) to generate two messengers: IP₃, which releases Ca²⁺ from intracellular stores via binding to specific receptors, and diacylglycerol (DAG), which activates various PKC isoforms. Increase in cytosolic Ca²⁺ results in activation of Ca²⁺/calmodulin-dependent MLC kinase and subsequent phosphorylation of MLC₂₀, an essential step in smooth muscle contraction (Murthy 2006; Deng, Ding et al. 2011). The selective m3 receptor antagonist, 4-DAMP, blocks acetylcholine-induced PI hydrolysis and initial muscle contraction.

Contractile agonists that activate G_q also activate G₁₃: both G proteins activate RhoA via distinct guanine nucleotide exchange factors (GEFs), and their involvement in smooth muscle is receptor-specific. Muscarinic m3 and CCK-A receptors activate RhoA via G_{α₁₃} only, whereas S1P₂ and motilin receptors activate RhoA via both G_{α_q} and G_{α₁₃} (Murthy, Zhou et al. 2001; Le

Page, Bi et al. 2003; Murthy, Zhou et al. 2003; Suzuki, Hajicek et al. 2009; Sabbatini, Bi et al. 2010). Activated RhoA, in turn stimulates Rho kinase, a serine/threonine protein kinase that belongs to family of enzymes including myotonic dystrophy protein kinase (DMP kinase), Cdc42-binding kinase, and citron kinase (Riento and Ridley 2003; Wansink, van Herpen et al. 2003). Rho kinase phosphorylates the regulatory subunit of MLC phosphatase, MYPT1, at Thr⁶⁹⁶ and inhibits catalytic activity resulting in increase in MLC₂₀ phosphorylation and sustained contraction (Fukata, Amano et al. 2001). ZIP kinase, a member of death-associated protein kinase family, was also shown to phosphorylate MYPT1 at Thr⁶⁹⁶, albeit with a greater potency, and also MLC₂₀ *in vitro* in a Ca²⁺-independent mechanism (Borman, MacDonald et al. 2002; Endo, Surks et al. 2004). However, the physiological significance of ZIP kinase *in vivo* is unclear. Stimulation of ZIP kinase activity by CCh and inhibition by the selective Rho kinase inhibitor, Y27632 provide evidence that the contractile agonist activate ZIP kinase and its activity is dependent on Rho kinase suggesting that Rho kinase is an upstream kinase. However, Rho kinase does not phosphorylate ZIP kinase *in vitro* suggesting that activation of ZIP kinase by Rho kinase may be indirect via one or more kinases (Graves, Winkfield et al. 2005). Carbachol-induced stimulation of Rho kinase and ZIP kinase activity and sustained contraction were also inhibited by 4-DAMP and Y27632, suggesting that sustained contraction was also exclusively mediated by m3 receptors. The results with Y27632 imply the involvement of Rho kinase pathway, but the relative roles of Rho kinase vs. ZIP kinase, in direct phosphorylation of MYPT1 to mediate contraction is unknown and awaits further studies with specific ZIP kinase inhibitors.

Muscarinic m2 receptors are coupled to inhibition of AC activity and this is consistent with the expression of AC V and VI isoforms in gastric smooth muscle cells. G_i proteins inhibit

AC V/VI isoforms and this is in contrast with the activation of AC II/IV forms that are not inhibited by G_i proteins (Murthy and Makhlouf 1997; Murthy and Makhlouf 1998; Wansink, van Herpen et al. 2003). Although m2 receptors are not directly coupled to muscle contraction, activation of these receptors is shown to augment m3 receptor-mediated contraction. This is partly due to inhibition of RGS4, an activator of $G\alpha_q$ -GTPase, and activation of G_{α_q} -dependent PLC- β 1 activity by m2-mediated stimulation of PI3-kinase activity (Hu, Li et al. 2009). The augmentation is attributed to inhibition of cAMP formation by m2 receptor activation. The physiological significance of decrease in cAMP formation was viewed as functional antagonism of on-going relaxation mediated by cAMP-dependent protein kinase (PKA) and facilitation of contraction via G_q/G_{13} -coupled pathways.

In conclusion, m3 receptors are coupled to stimulation of PI hydrolysis, and Rho kinase and ZIP kinase activity, and muscle contraction, whereas m2 receptors are coupled to inhibition of AC activity in gastric smooth muscle.

IV. 2. Regulation of m2 and m3 receptor signaling by caveolae and caveolin-1.

Caveolae and their main membrane proteins, caveolins, concentrate receptors, G proteins, effector enzymes, and associated signaling molecules so as to facilitate coordinated generation and degradation of second messengers. Caveolins contain a caveolin-scaffolding domain that binds signaling molecules via a characteristic caveolin-binding domain in these molecules. Caveolae contain abundant cholesterol and sphingolipids and the cholesterol is necessary for caveolae structure and function. Depletion of cholesterol with pharmacological treatments alters signaling by both tyrosine kinase- and G protein-coupled receptors (GPCRs) (Chini and Parenti 2004; Matthews,

Taggart et al. 2005; Patel, Murray et al. 2008). The effect of caveolae and caveolins on smooth muscle contraction appears to be receptor- and ligand-specific. In general caveolae and caveolins appear to facilitate vascular smooth muscle contraction via G protein coupled receptors, and inhibit proliferation via tyrosine kinase-coupled receptors (Insel, Head et al. 2005; de Laurentiis, Donovan et al. 2007). The effect of caveolae and caveolins on muscarinic m3 and m2 receptor-mediated signaling is not clear.

In the present study, the role of caveolae/caveolin-1 in the regulation of m2 and m3 receptor signaling was analyzed using pharmacological (M β CD), molecular (caveolin-1 siRNA) and genetic (caveolin-1 knockout) approaches. A novel aspect of these studies is that similarity of results obtained with all three approaches. M β CD, a water-soluble heptasaccharide, binds cholesterol with high specificity and has been used extensively as cholesterol depleting pharmacological agent. Depletion of cholesterol by M β CD results in disruption of caveolae and affects plasma membrane signaling pathways. The exact mechanism by which cholesterol removal affects signaling pathways is not clear, however, cholesterol replenishment restores the plasma membrane signaling (Ostrom and Insel 2006). The efficiency and specificity of M β CD treatment was confirmed in vascular and airway smooth muscle by the significant decrease in caveolae and lack of effect of M β CD on other cellular structures (Gosens, Stelmack et al. 2007). The intact depolarization-induced response of M β CD treated cells to KCl also excluded the non-selective damage of the plasma membrane (Dreja, Voldstedlund et al. 2002). Although use of M β CD and other cholesterol chelating drugs remains a mainstay to study the function of caveolae, the effects of M β CD could be non-specific than molecular approaches. Disruption of caveolae by M β CD can affect signaling by redistribution of proteins, and thus changing their relative local concentrations or by changing the chemical environment of the membrane. To more precisely understand the role of caveolin-1, the most

abundant isoform of caveolin in smooth muscle, we used the caveolin-1 siRNA approach to suppress caveolin-1 expression and to examine the specific role of caveolin-1 in muscarinic signaling. Transfection of siRNA was analyzed by immunocytochemistry and suppression of caveolin-1 expression was analyzed by western blot. We also took the advantage of using caveolin-1 KO mice as they became available commercially. Caveolin-1 KO mice have provided excellent animal models to examine the physiological significance of caveolae. These mice have been used in previous studies to examine the role of caveolin-1 in the regulation of vascular and visceral muscle function (Zhao, Liu et al. 2002; Schwencke, Schmeisser et al. 2005; Insel and Patel 2007; Sathish, Yang et al. 2011). Despite the importance of caveolin-1 in the formation of caveolae and signal transduction, caveolin-1 KO mice are viable raising the possibility of compensatory mechanisms.

Our studies indicate that the effect of caveolae and caveolin-1 on muscarinic receptor signaling appears to be receptor-and signaling-specific. Caveolae and caveolin-1 facilitate G_q/G_{13} -coupled m3 receptor signaling, but not G_i -coupled m2 receptor signaling. In contrast, caveolae and caveolin-1 facilitate m2 receptor, but not m3 receptor internalization.

IV. 2a. Regulation of G_q -coupled m3 receptor signaling by caveolin-1.

Carbachol-induced stimulation of PI hydrolysis in the presence of methoctramine was measured as readout of G_q -coupled m3 receptor signaling. Carbachol-induced PI hydrolysis was significantly inhibited by the disruption of caveolae in freshly dispersed cells and by the suppression of caveolin-1 in cultured muscle cells. Similar decrease in PI hydrolysis was obtained in gastric muscle from caveolin-1 KO mice. These results suggest that caveolin-1 positively regulates G_q -coupled m3 receptor signaling. This could lead to decrease in IP_3

formation, IP_3 -induced Ca^{2+} release, and Ca^{2+} /calmodulin-dependent MLC kinase activity, MLC_{20} phosphorylation and muscle contraction in response to CCh. Consistent with this notion, CCh-induced MLC_{20} phosphorylation and contraction was significantly reduced in cells treated with M β CD. Similar decrease in contraction was also observed in muscle cells obtained from caveolin-1 KO mice. These results are similar to recent data from studies with vascular and airway smooth muscle cells, where disruption of caveolae with M β CD leads to reduced contraction in response to contractile agonists such as endothelin-1, serotonin, $\alpha 1$ adrenergic receptor agonist, and acetylcholine (Cristofaro, Peters et al. 2007; Gosens, Stelmack et al. 2007). However, studies by Shakirova et al. showed that the effect of caveolin-1 was agonist- and tissue-specific. Response of ileum longitudinal muscle to endothelin-1, but not to carbachol or serotonin was reduced in caveolin-1 KO mice, whereas the response of femoral arterial muscle to $\alpha 1$ -adrenergic agonist was increased (Okamoto, Schlegel et al. 1998; Shakirova, Bonnevier et al. 2006).

Collectively, our results provide conclusive evidence that caveolin-1 facilitates G_q -coupled m3 receptor signaling and muscle contraction in response to contractile agonists such as CCh. Although, it is not clear whether caveolin-1 targets m3 receptor, or $\text{G}\alpha_q$ or PLC- $\beta 1$ isoform to facilitate the effect. Analysis of m3 receptor expression in sucrose density gradient fractions showed that m3 receptors are not associated with caveolin-1. This suggests that distal components of m3 receptor may be spatially associated with caveolin-1. Previous studies have shown that $\text{G}\alpha_q$ protein contain caveolin binding motif and directly interacts with caveolin-1 proteins (Sengupta, Philip et al. 2008; Allen, Yu et al. 2009; Guo, Golebiewska et al. 2011). Thus, it is possible that the interaction of $\text{G}\alpha_q$ with caveolin-1 augments PI hydrolysis. In support to this notion, PI hydrolysis in response to substance P, which interacts with G_q -coupled

neurokinin receptor, was also inhibited by M β CD. It is also important to note that in smooth muscle caveolae contains proteins that are not only important for GPCR signaling, but also ion channel and pumps involved in Ca²⁺ homeostasis. Recent studies demonstrated that KCl-induced contraction was similar in wild type and caveolin-1 KO mice suggesting that caveolin-1 KO does not affect the membrane-depolarization process (Shakirova, Bonnevier et al. 2006).

In conclusion, the results described here indicate a significant role for caveolin-1 in regulation of G_q-coupled PLC- β 1 activity and muscle function. Further work examining the localization of G α_q proteins and PLC- β 1 isoforms in caveolae and their interaction with caveolin-1 may reveal more precise understanding of the mechanism regulating PI hydrolysis and contraction.

IV. 2b. Regulation of G₁₃-coupled m3 receptor signaling by caveolin-1.

Stimulation of Rho kinase and ZIP kinase activity in response to CCh was measured as readouts of G₁₃-coupled m3 receptor signaling. Previous studies in intestinal smooth muscle have shown that acetylcholine-stimulated Rho kinase activity is downstream of G₁₃/RhoA signaling (Buyukafsar and Levent 2003; Murthy, Zhou et al. 2003; Rattan, Puri et al. 2003). Our studies provide evidence that contractile agonists such as carbachol also stimulate ZIP kinase activity and its activation is downstream of Rho kinase.

Carbachol-stimulated Rho kinase and ZIP kinase activity was significantly inhibited by the disruption of caveolae in freshly dispersed cells and by the suppression of caveolin-1 in cultured muscle cells. Similar decrease in Rho kinase and ZIP kinase activity was obtained in gastric muscle from caveolin-1 KO mice. One of the downstream targets of Rho kinase and ZIP kinase is MYPT1. Both kinases phosphorylate MYPT1 at Thr⁶⁹⁶ leading to inhibition of MLC

phosphatase, increase in MLC₂₀ phosphorylation and muscle contraction. Thus, inhibition of Rho kinase and ZIP kinase activity could lead to decrease in MYPT1 phosphorylation and muscle contraction in response to CCh. Consistent with this notion, CCh-induced MYPT1 phosphorylation at Thr⁶⁹⁶ and contraction were significantly reduced in cells treated with MβCD. Similar decrease in contraction was also observed in muscle cells obtained from caveolin-1 KO mice. Thus, our results suggest that caveolin-1 is necessary for optimal G₁₃/RhoA-dependent signaling in gastric smooth muscle. It is not clear, however, whether caveolin-1 targets RhoA or Rho kinase to facilitate the effect. Lack of m3 receptors in the caveolar fractions suggests that distal components of m3 receptor such as RhoA may be spatially associated with caveolin-1. RhoA is small GTP binding proteins and exists in active GTP-bound and inactive GDP-bound states. The inactive cytosolic pool is associated with Rho-dissociation inhibitor and the active plasma membrane pool is associated with caveolae. Previous studies by confocal microscopy in mesenteric arteries showed activation-induced colocalization of RhoA with caveolin-1, and MβCD treatment significantly decreased the colocalization and contraction (Dubroca, Loyer et al. 2007). Similar inhibition of agonist-induced RhoA translocation and contraction was obtained by blocking caveolin-1 scaffolding domain with a synthetic peptide in ferret aorta (Taggart, Leavis et al. 2000). In addition, sites of monoglycosylation and ADP ribosylation of RhoA, which inhibits RhoA translocation and muscle contraction, are located within the caveolin-1 binding motif of RhoA (Dubroca, Loyer et al. 2007). Although, caveolin-1 is generally considered as negative regulator of signaling molecules, our studies using several complimentary approaches suggested that caveolin-1 positively regulates G₁₃/RhoA-mediated signaling by m3 receptors. The mechanisms governing the facilitation of RhoA activation by caveolin-1 are unknown. Activation of RhoA is dependent on the Rho guanine exchange factors (RhoGEFs).

There are several members in the RhoGEF family of proteins that include p115RhoGEF, PDZ-RhoGEF, and LARG (*leukemia associated RhoGEF*). All the RhoGEF proteins harbor a common signature motif including RGS (regulator of G protein signaling), Db1 homology (DH), and pleckstrin homology (PH) domains that are responsible for the exchange of GDP for GTP after binding to inactivated G_{α} subunits. The role of RhoGEFs can be described that of a molecular link between heterotrimeric G proteins and monomeric G proteins. Further experiments are needed to identify the localization of RhoA and specific Rho GEFs involved in the activation of Rho kinase and ZIP kinase and muscle contraction in gastric muscle.

It is important to note that downstream of m3 receptor activation, PKC, in addition to Rho kinase and ZIP kinase, play an important role in the inhibition of MLC phosphatase and regulation of muscle contraction. In the active GTP-bound conformation, RhoA physically interact with downstream effectors such as Rho Kinase and phospholipase D (PLD) and stimulates their activity. Phosphatidic acid formed as a hydrolytic product of phosphatidylcholine by PLD activity, leads to sustained activation of several PKC isozymes (Murthy, Grider et al. 2000; Preininger, Henage et al. 2006). PKC, in turn, phosphorylates an endogenous MLC phosphatase inhibitor known as CPI-17 and phosphorylation greatly accelerates the ability of CPI-17 to inhibit MLC phosphatase and augment MLC₂₀ phosphorylation (Li, Eto et al. 1998; Woodsome, Eto et al. 2001; Tazzeo, Bates et al. 2012). We did not examine the effect of caveolin-1 on this PKC/CPI-17 pathway in gastric muscle. Previous studies in vascular muscle have identified caveolae as regulators of PKC signaling. Studies by Je et al (Je, Gallant et al. 2004) showed that chemical loading of caveolin-1 peptide that inhibits caveolin-1 function caused significant inhibition of contraction in response to phorbol esters. Understanding agonist-induced, isoform(s)-specific translocation of PKC could lead to assess the singular contribution

of Rho kinase and ZIP kinase pathway to the decrease in muscle contraction in cells treated with M β CD or caveolin-1 siRNA or in cells isolated from caveolin-1 KO mice.

In conclusion, the results described here indicate a significant role for caveolin-1 in regulation of G₁₃-coupled RhoA-dependent pathway and muscle function. Further work examining the localization of RhoA and PKC isoforms in caveolae and their interaction with caveolin-1 may reveal more precise understanding of the mechanism regulating Rho kinase/ZIP kinase and sustained muscle contraction.

IV. 2c. Regulation of G_i-coupled muscarinic m2 receptor signaling by caveolin-1.

Since m2 receptors are coupled to PTx-sensitive G_i proteins, inhibition of forskolin-stimulated cAMP formation, reflecting inhibition of AC activity was measured as readout of G_i-coupled m2 receptor signaling. The effect of caveolae and caveolin-1 was analyzed using all three approaches described above. In contrast to the effect of caveolae and caveolin-1 on the G_q/G₁₃-coupled m3 receptor signaling, disruption of caveolae with M β CD in freshly dispersed muscle cells or suppression of caveolin-1 in cultured muscle cells with caveolin-1 siRNA did not affect the inhibition of AC activity in response to CCh. Similar results were obtained using caveolin-1 KO mice. CCh-induced inhibition of AC activity was similar in gastric muscle cells from WT mice and caveolin-1 KO mice. The similarity of results with three distinct approaches strongly suggests that caveolin-1 has no effect on G_i-coupled m2 receptor signaling in gastric muscle cells.

Taken together, our studies provide evidence that caveolin-1 differentially regulate m3 and m2 receptor signaling in gastric muscle: m3-coupled G α_q and G α_{13} signaling is facilitated, whereas m2-coupled G α_i signaling is not affected.

Our studies also demonstrated that the increase in AC activity in response to direct activator forskolin was similar in control cells and in cells treated with M β CD or caveolin-1 siRNA. Stimulation of AC activity by forskolin was also similar in gastric muscle from WT mice and caveolin-1 KO mice. These results suggest that caveolin-1 had no effect on AC activity. Our results differ with previous studies showing suppression of caveolin-1 in C6 glioma cells or lack of caveolin-1 in the brain of caveolin-1 KO mice significantly augmented forskolin-stimulated AC activity (Allen, Yu et al. 2009; Head, Hu et al. 2011). These studies suggest that the effect of caveolin-1 on AC activity varies within different tissues and/or with the expression of specific AC isoforms. There are nine different isoforms of AC's and their localization in caveolae seems to be isoforms-specific. AC3, AC5, AC6 and AC8 are expressed in lipid rafts while the other isoforms of AC are not (Ostrom, Liu et al. 2002). Localization of the AC isoforms in membrane microdomains play an important role in the coupling of G protein coupled receptors to discrete AC isoforms and differential regulation of AC isoforms by signaling molecules such as Ca²⁺ and PKC. Further experiments are sought to characterize the localization of AC isoforms in the caveolae to define the regulation of their activity by signaling molecules. In addition, given that cAMP signaling is compartmentalized by the expression of phosphodiesterases, we speculate that such intermolecular association with other signaling partners in caveolar microdomains is also relevant to understand the role of caveolin-1 in cAMP signaling.

In conclusion, the results described here indicate that m2 receptor signaling via G_i is not regulated by caveolae and caveolin-1 and this is in contrast to the positive regulation of m3 receptor signaling via G_{q/13}. Further work examining the localization of AC isoforms and cAMP-specific phosphodiesterases, and their regulation by signaling molecules may reveal more precise understanding of the mechanisms involved in cAMP signaling in gastric smooth muscle.

IV. 3. Regulation of m2 and m3 receptor internalization by caveolae and caveolin-1.

Compelling evidences showing selective regulation of m3 receptor signaling by caveolin-1 necessitated the importance to look into the probability of occurrence of muscarinic receptors in caveolar microdomains. Western blot analysis of m2 and m3 receptors in different fraction of sucrose density centrifugation showed that in basal state both m2 and m3 receptors are absent in caveolar fraction. However, upon treatment with CCh, there was translocation of m2 but not m3 receptors to caveolar fractions raising the possibility that caveolae could facilitate m2, but not m3 receptor internalization.

Multiple “turn off” signals operate in the cell to fine-tune the intensity of G protein coupled receptor signaling. The most important mechanism involved regulating the density of surface receptors through the process of receptor desensitization and subsequent internalization. Receptor desensitization can be broadly classified into two major categories: homologous desensitization that strictly target the agonist-bound receptors leaving the unbound non-activated receptor population to function, and heterologous desensitization that target ligand-free receptors. Homologous desensitization of agonist-occupied receptors is initiated by GRK (G protein coupled receptor kinase)-mediated phosphorylation of the receptor and binding of the scaffolding protein β -arrestin to the phosphorylated receptor, which uncouples the receptor from the G protein as a prelude to receptor internalization (Delom and Fessart 2011).

The molecular events that drive the receptor internalization process can be either clathrin-dependent or clathrin-independent, caveolae-dependent. Internalization via the clathrin-dependent pathway triggers activation of signaling pathways (e.g., ERK1/2 and Src kinase) from early endosomes, whereas internalization via caveolae results in accelerated receptor degradation. Although the importance of clathrin-dependent internalization in receptor

trafficking and activation of signaling pathways for several GPCRs is well established, the contribution of the caveolar-dependent pathway has been little explored. In clathrin-dependent model, binding of β -arrestins provides a scaffold for associating the receptor to clathrin and facilitates the formation of clathrin-coated pits. As the clathrin pits gets matured, they are invaginated and pinch off from the cell surface by the GTPase activity of dynamin and progressively released into the cytoplasm as free clathrin coated vesicles and the clathrin coat is removed (Bunemann, Lee et al. 1999). In the clathrin-independent, caveolar-dependent model, internalization of specific receptors through caveolae is strictly dependent on tyrosine kinase signaling. Ligand binding triggers receptor clustering in caveolar microdomains and activation of phosphorylation events. The essential step in the internalization process via caveolae involves activation of Src kinase following receptor clustering. Activation of Src causes phosphorylation of caveolar coat proteins such as caveolin-1 at Tyr¹⁴ (Sverdlov, Shajahan et al. 2007). The primary function of Src mediated phosphorylation of caveolar proteins is to regulate and stabilize the assembly of multi-protein complexes that finally leads to caveolae fission and internalization. Ectopic expression of phosphorylation-deficient caveolin-1 (Y14F) inhibits agonist-induced internalization of GPCRs via caveolae-dependent pathways (Shajahan, Timblin et al. 2003).

IV. 3a. Regulation of m2 receptor internalization by caveolae and caveolin-1.

Internalization was measured as decrease in radioligand ($[^3H]$ Scopolamine) binding to surface receptors after treatment of cells with CCh for 20 min. Treatment of cells with CCh for 20 min significantly inhibited the binding of $[^3H]$ Scopolamine, measured in the presence of 4-DAMP, suggesting internalization of m2 receptors. Previous studies with radioligand binding studies showed that 20-min treatment is sufficient to cause maximum internalization of various

GPCRs. The role of caveolin-1 in the regulation of m2 and m3 receptor internalization was examined using M β CD in freshly dispersed muscle cells and in cultured muscle cells transfected with caveolin-1 siRNA. Limited yield of muscle cells from mice precluded the use of caveolin-1 KO mice for binding studies. Disruption of caveolae or suppression of caveolin-1 had no effect on the maximal binding of m2 receptors, but significantly attenuated m2 receptor internalization. Internalization of m2 receptors was also attenuated by the blockade of Src kinase. These results suggest that m2 receptor internalization was facilitated by caveolae or caveolin-1 and consistent with the translocation of m2 receptors to caveolar fractions. This is in contrast to the lack of effect of caveolin-1 on the rapid responses to m2 receptor activation such as inhibition of AC activity. In our experiments, role of caveolin-1 in m2 receptor recycling or endocytosis remains to be clarified. Future studies will also be necessary to identify signaling molecules involved in the activation of Src kinase by m2 receptors and regulation of m2 receptor internalization via the caveolar pathways. Previous studies demonstrated that activation of Src kinase via G $\beta\gamma$ is critical for internalization via caveolar pathways (Murthy, Mahavadi et al. 2008). These studies linked G $\beta\gamma$ signaling to the regulation of caveolae-mediated internalization and demonstrated that Src kinase is the downstream effector of the G $\beta\gamma$ heterodimer. Stimulation of G $_i$ -coupled m2 receptors is shown to activate Src kinase in gastrointestinal muscle (Murthy 2008), however, further studies are sought to understand the role of G $\beta\gamma$ in the activation of Src kinase and m2 receptor internalization via caveolae-dependent pathways in gastric muscle.

IV. 3b. Regulation of m3 receptor internalization by caveolae and caveolin-1.

Treatment of cells with CCh for 20 min significantly inhibited the binding of [3 H]Scopolamine, measured in the presence of methoctramine, suggesting internalization of m3 receptors. The extent (~ 70%) of internalization of m3 receptors is similar to the m2 receptor internalization. Disruption of caveolae or suppression of caveolin-1 had no effect on the maximal binding of [3 H]Scopolamine to m3 receptors and did not affect m3 receptor internalization. These results suggest that m3 receptor internalization is caveolae-independent and probably involves clathrin-dependent pathways. This is contrast to the positive effect of caveolin-1 on the rapid responses of m3 receptor signaling such as stimulation of PI hydrolysis, Rho kinase and ZIP kinase activity. These results also suggest that caveolae-dependent internalization is receptor-specific. Similar, caveolae-dependent pathway for internalization of vasoactive intestinal peptide receptor, VPAC₂ was obtained in gastric muscle (Muthy, unpublished).

Caveolin-binding motifs are present in many G protein coupled receptors (GPCRs) such as β -adrenergic receptor, endothelin receptors, muscarinic receptors, opioid receptors, and mGlu receptor 1 α (Chini and Parenti 2004; BM 2012). Desensitization in response to angiotensin II in rat aorta was inhibited by M β CD suggesting that AT₁ receptor internalization occurs via caveolae-dependent pathways. Caveolin-1 was coimmunoprecipitated with AT₁ receptor after agonist stimulation, and this interaction was inhibited by M β CD (Linder, Thakali et al. 2007). Consensus caveolin binding motifs are also present in GRKs (G-protein receptor kinases) and interaction of GRKs with caveolin play an important role in agonist-induced internalization of GPCRs (Carman, Lisanti et al. 1999). In addition to GPCR internalization, tyrosine kinase-coupled receptors are internalized via caveolae-dependent pathway (Mukherjee, Tessema et al.

2006). Caveolin-1 is a target of insulin-like growth factor I (IGF-I) receptor and plays a role in IGF-I receptor internalization and signaling. IGF stimulates caveolin-1 tyrosine phosphorylation and the IGF-I receptors co-localize with the caveolin-1 in the plasma membrane (Salani, Passalacqua et al. 2010).

In conclusion, our studies provide evidence that caveolin-1 positively regulates m2, but not m3 receptor internalization in gastric muscle. This is in contrast to the regulation by caveolin-1 of m2 and m3 receptor mediated rapid signaling pathways.

VI. 4. Regulation of Excitation-transcription coupling by caveolin-1

Unlike other cell types, such as cardiac and skeleton muscle cells smooth muscle (SM) cells do not undergo terminal differentiation, rather they switch between proliferative and differentiated phenotypes in response to various intrinsic and extrinsic signals. SM cells derive their characteristic contractile and physiological phenotype through the transcriptional activation of a distinct subset of SM-specific genes encoding for proteins such as SM-myosin heavy chain, SM- α actin, γ -actin, SM-myosin light chain kinase, calponin, and caldesmon (Wang and Olson 2004).

Serum response factor (SRF) a member of the MADS (*MCMI*, *Agamous*, *Deficiens*, *SRF*) family of transcription factors plays an important role in the regulation of SM gene expression and differentiation (Shore and Sharrocks 1995). SRF recognizes a consensus sequence of CC(A/T)₆GG, also known as the CArG box or serum response element on the target genes to drive their transcription (Kim, Ip et al. 1997; Manabe and Owens 2001). SRF associate with other transcription factors aptly termed as co-factors to control and regulate the expression of SRF target genes. Myocardin constitute one of the most intimate coactivators of SRF that was

originally identified in a bioinformatics screen of cardiac gene expression profiling, and its expression is specific for cardiac myocytes and SM cells (Wang, Chang et al. 2001). Myocardin binds and activate promoters containing multiple CArG elements along with stimulated SRF. The myocardin family of proteins contains two additional members and shares homology with myocardin-related transcription factor A (MRTF-A) and factor B (MRTF-B) (Wang, Li et al. 2002). The critical role of myocardin and its family in SMC differentiation comes from the finding that showed that the differentiation process is severely compromised when dominant mutant form of myocardin and MRTFs or small interfering RNA targeted to myocardin was used to suppress its activity (Yoshida, Sinha et al. 2003).

SRF/myocardin-mediated transcription of SM marker genes is regulated by actin dynamics and a decrease in G actin pool is critical for SRF-dependent activation of SM-specific genes (Copeland and Treisman 2002). Recently, Ca^{2+} and Rho family of small GTPases including RhoA has been shown to facilitate F-actin to G-actin ratio through multiple pathways that finally stimulate SRF activity (Wamhoff, Bowles et al. 2004). Overexpression of constitutively active RhoA stimulates transcription of SRF/myocardin-mediated genes such as SM- α actin, SM-MHC and the stimulation is dependent on Rho kinase activity. In addition, increase in cytosolic Ca^{2+} upregulates the expression of myocardin (Mack, Somlyo et al. 2001). These results suggest that the factors that regulate Ca^{2+} and RhoA-dependent pathways could modulate SM marker genes (excitation-transcription) long-term, in addition to modulating muscle contraction (excitation-contraction) short-term as shown above. Positive regulation of Ca^{2+} and RhoA signaling by caveolae and caveolin-1 prompted us to examine the role of caveolin-1 in the regulation of smooth muscle contractile phenotype. The present study significantly extends understanding the role of caveolin-1 in the regulation of smooth muscle

marker genes because our results demonstrate that caveolin-1 suppresses the proliferative signals and facilitates differentiated contractile phenotype in gastric smooth muscle. Our data reveal that caveolin-1 represses EGF receptor and ERK1/2 activity and promotes the expression of contractile proteins and their transcription factors. Suppression of caveolin-1 attenuated expression of SRF and myocardin and SM contractile proteins such as γ -actin and caldesmon, and augmented basal and agonist-induced EGF receptor phosphorylation and ERK1/2 activity. Mitogen-activated protein kinases such as ERK1/2 are recognized as important signaling molecules in cell proliferation.

Our results are in line with previous studies indicating that vascular muscle cells, fibroblasts and epithelial cells isolated from caveolin-1 KO mice exhibit hyperproliferative phenotype (Cohen, Park et al. 2003). Studies in airway and vascular muscle demonstrated that expression of caveolin-1 was more than two fold higher in quiescent contractile muscle cells compared to proliferating muscle cells in culture (Gosens, Stelmack et al. 2011). Overexpression of caveolin-1 causes antiproliferative effects. Consistent with the antiproliferative role of caveolins, expression of receptors for growth factors such as EGF and PDGF, and constituent of growth factor signaling such as Src, Shc, Grb₂ and Ras are localized in caveolae (Gosens, Stelmack et al. 2006). Interaction of these signaling proteins with caveolins suppresses their activity. Transactivation of growth factor signals by GPCRs is also associated with the decrease in the binding of growth factor receptors with caveolin-1 (Olivares-Reyes, Shah et al. 2005), however, the molecular mechanisms that causes disassociation of caveolin-1 from growth factor signal are not clear. In addition, it was demonstrated that non-phosphorylated, but not phosphorylated (activated) EGF receptor is associated with caveolin-1 (Matveev and Smart 2002). Caveolin-1 KO mice do not develop tumors spontaneously, but are more susceptible to

tumor formation in response to carcinogens (Torres, Tapia et al. 2006). Thus, there is clear association between caveolae and growth factor signal. Our current findings in gastric muscle are highly reminiscent of these observations, and reveal the importance of caveolin-1 in regulating proliferative and contractile phenotype in gastrointestinal smooth muscle. It is interesting to note that the smooth muscle differentiation is not affected in caveolin-1 KO mice, suggesting functional compensation for the lack of caveolin-1.

Although, our studies suggest a link between decrease in Ca^{2+} and RhoA signaling in the absence of caveolin-1 to decrease in contractile protein expression, they do not exclude the existence of additional, transcriptional and post-translational mechanisms downstream of caveolin-1 that control contractile proteins expression. Preliminary studies from our laboratory, using microarray analysis, compared of microRNA levels from gastric muscle expressing or not expressing caveolin-1. One of the striking changes detected in response to absence of caveolin-1 expression was upregulation of microRNA133a. MicroRNAs play an important role in the regulation of gene expression by pairing to the mRNA targets. Searching for conserved targets of microRNA-133a by RNA hybrid analysis, we identified the transcription factor SRF, which regulates expression of contractile proteins. SRF expression is negatively regulated by microRNA-133a. In vascular smooth muscle, overexpression of microRNA-133a reduced SRF expression, whereas the effects were opposite when microRNA-133a was inhibited by anti-microRNA-133a. Future studies are important to examine the mechanisms involved in the regulation of contractile protein expression by caveolin-1 in gastric smooth muscle involving microRNA-133a. It is also important to examine the expression profile of caveolins in gastrointestinal disorders with smooth muscle hyperproliferation. If caveolin-1 expression is

decreased, this could contribute to the smooth muscle proliferation and stricture formation in inflammatory diseases.

***In conclusion,** our results provide evidence that caveolin-1 negatively regulates proliferative phenotype and positively regulates contractile phenotype in gastric muscle. Further studies examining the molecular mechanisms involved in the regulation of contractile protein expression by caveolin-1 may reveal more precise understanding of the mechanism regulating SM contractile phenotype.*

In summary, our studies demonstrated that CCh, acting via m3 receptors caused stimulation of PI hydrolysis and RhoA-dependent signaling (Rho kinase and ZIP kinase) and induced muscle contraction, and acting via m2 receptors caused inhibition of AC activity. Stimulation of PI hydrolysis, RhoA-dependent signaling and muscle contraction were attenuated in dispersed cells by the disruption of caveolae with M β CD, or by the suppression of caveolin-1 in cultured muscle cells transfected with caveolin-1 siRNA. Similar inhibition of PI hydrolysis and RhoA-dependent signaling was obtained in gastric muscle from caveolin-1 KO mice compared to control mice. Inhibition of cAMP formation via m2 receptors, in contrast, was not affected in dispersed cells treated with M β CD or in cultured muscle cells transfected with caveolin-1 siRNA. Similarly, inhibition of AC activity was not affected in gastric muscle from caveolin-1 KO mice. In the basal state both m2 and m3 receptors are present in non-caveolar fractions and upon treatment with CCh, m2, but not m3 receptors are translocated to caveolar fractions. Pretreatment of muscle cells with CCh caused internalization of both m2 and m3 receptors: internalization of m2, but not m3 receptors was blocked in dispersed cells treated with M β CD or in cultured muscle cells transfected with caveolin-1 siRNA. The possible physiological

significance of this regulation of m2 receptor internalization is not clear. However, there exists the potential for regulation of m3 receptor signaling by m2 receptors; this should be investigated more rigorously. Thus, caveolin-1 selectively regulates m3/G_q-mediated PI hydrolysis, m3/G₁₃-mediated RhoA signaling and contraction, and m2 receptor internalization. Since, there was no change in the binding of [³H]Scopolamine to m2 and m3 receptors by caveolae and caveolin-1, we propose that caveolae and caveolin-1 promote m3 receptor signaling via enhanced receptor-effector coupling rather than enhanced receptor affinity. Furthermore, expression of contractile proteins, γ -actin and caldesmon and the transcription factors SRF and myocardin that mediate the expression of contractile proteins are down regulated in cells transfected with caveolin-1 siRNA suggesting that caveolin-1 expression is also important for the maintenance of smooth muscle contractile phenotype. The study also suggests that caveolin-1 could be a marker of the mature, contractile smooth muscle cell phenotype, and is a determinant of functional characteristics of gastric muscle. In parallel, caveolin-1 suppresses the activity of proliferative signals.

In conclusion our studies using pharmacological, molecular and genetic approaches, provide conclusive evidence that caveolae and caveolin-1 play an important role in orchestrating G protein coupled receptor signaling to have dual pro-excitation-contraction and excitation-transcription coupling, and anti-proliferative role in gastric smooth muscle.

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VITA

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Education

Master of Science (2006)

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Thesis/Dissertation

Regulation of excitation-contraction and excitation-transcription coupling in gastrointestinal smooth muscle by caveolin-1.

Key Areas of Research Experience

- | | |
|---|--|
| <ul style="list-style-type: none">• Cell culture | <ul style="list-style-type: none">• Cell signalling techniques (Radioactivity) |
| <ul style="list-style-type: none">• Cell and molecular Biology | <ul style="list-style-type: none">• Confocal Microscopy |
| <ul style="list-style-type: none">• Biochemical techniques | <ul style="list-style-type: none">• Immuno-histochemistry |
| <ul style="list-style-type: none">• siRNA | <ul style="list-style-type: none">• Basic electrophysiology |
| <ul style="list-style-type: none">• Ligand binding studies | <ul style="list-style-type: none">• Ca²⁺ imaging (Fura dyes) |
| <ul style="list-style-type: none">• ELISA | <ul style="list-style-type: none">• Two electrode voltage clamp |
| <ul style="list-style-type: none">• Micrometry to measure smooth muscle contraction | <ul style="list-style-type: none">• Protein expression and purification |
| <ul style="list-style-type: none">• Isolation of smooth muscle cells | <ul style="list-style-type: none">• Animal Handling |
| | <ul style="list-style-type: none">• <i>In vivo</i> gastric emptying studies |

Experience

GRADUATE RESEARCH ASSISTANT

August 2008-present

TEACHING EXPERIENCE

Human Physiology Lab, 2011

Advance coursework

- Cellular Signalling, Fall 2010
- Cell signalling and growth control. Spring 2011
- Techniques in molecular biology and genetics, Fall 2009

Fellowships/Awards

1. Nominated member of Phi Kappa Phi Society of VCU's School of Medicine, 2012

2. INDO-US Research Internship in Science and Technology, 2011-12

3. Award for **best oral presentation**, Physiological society of India, 2011

4. Best Poster Award, Graduate School Symposium, VCU, 2011

5 Virginia Commonwealth University Teaching Assistant Award, 2008

6. Lal Chand Mukherjee International Scholarship, University of Calcutta, 2008

Publications

1. Sayak Bhattacharya, Sunila Mahavadi, Othman Al-Shboul, Senthilkumar Rajagopal, John R. Grider. Karnam S. Murthy. Differential regulation of muscarinic m2 and m3 receptor signalling in gastrointestinal smooth muscle by caveolin-1. Manuscript submitted under review.

2. Sayak Bhattacharya, Sunila Mahavadi, Ruiszhe Zhou, John R. Grider, Karnam S. Murthy. The role of caveolin-1 in the excitation-transcription coupling in gastrointestinal smooth muscle. Manuscript under review.

3. Senthilkumar Rajagopal, Divya P. Kumar, Sunila Mahavadi, Sayak Bhattacharya, Ruizhe Zhou, Carlos Corvera, Nigel W. Bunnett, John R. Grider and Karnam S. Murthy. Activation of G protein-coupled bile acid receptor, TGR5 induces smooth muscle relaxation via PKA/Epac-mediated inhibition of RhoA/Rho kinase pathway. Manuscript submitted under review.

4. Rashad Ahmed, Sunila Mahavadi, Othman Al-Shboul, Sayak Bhattacharya, John R. Grider, Karnam S. Murthy. Characterizing the signalling pathways coupled to melatonin receptors in gastrointestinal smooth muscle. Manuscript submitted under review.

Poster Presentation

1. Sayak Bhattacharya, O. Al-Shboul, S. Mahavadi, J. R. Grider and K. S. Murthy. Role of Rho-A dependent sustained contraction by caveolin-1 in gastric smooth muscle. Experimental Biology, 2011.

2. Sayak Bhattacharya, S. Mahavadi, J. R. Grider and K. S. Murthy. Caveolin-1 differentially regulates muscarinic m3 and m2 receptor signalling in gastric smooth muscle. Experimental Biology, 2011.

3. O. Al-Shboul, Sayak Bhattacharya, S. Mahavadi, S. Rajagopal, R. Zhou, K. S. Murthy. Agonist induced Rho Kinase and ZIP kinase activity in different regions of the stomach. Experimental Biology, 2011.

4. V.R. Joginapally, S. Rajagopal, Sayak Bhattacharya, R. Zhou. PCR amplification of a novel transcription initiation factor gene (TL-1) in smooth muscle cells of rabbit gastrointestinal tract and its phylogenetic analysis. Experimental Biology, 2011.

5. Sayak Bhattacharya, S. Rajagopal, S. Mahavadi, R. Zhou, J. R. Grider, and K. S. Murthy. Regulation of muscarinic m2 and m3 receptor signaling and contractile protein expression by caveolin-1 in gastrointestinal smooth muscle. Keystone Symposia, Canada, 2012.

6. Sayak Bhattacharya, S. Mahavadi, R. Zhou, J.R. Grider and K.S. Murthy. Regulation of excitation-contraction and excitation-transcription coupling by caveolin-1 via microRNA-133a and transcription factors, FOX4 and Elk1 in gastrointestinal smooth muscle. Neurogastroenterology meeting, Bologna, Italy, 2012.

7. S. Rajagopal, D.P. Kumar, Sayak Bhattacharya, S. Mahavadi, R. Zhou, C. Corvera, N. Bunnett, J.R. Grider, and K.S. Murthy. Activation of G protein-coupled bile acid receptor, TGR5 induces muscle relaxation via PKA- and Epac-mediated inhibition of RhoA/Rho kinase pathway. Keystone Symposia, Canada, 2012.

8. S. Rajagopal, D.P. Kumar, Sayak Bhattacharya, J.R. Grider, and K.S. Murthy. Characterization of G_z-coupled dopamine D3 receptors in gastric smooth muscle. Experimental Biology, 2012.

Memberships/Scholarly Societies

AMERICAN PHYSIOLOGICAL SOCIETY